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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 15/53, 9/02, C12Q 1/26	A1	(11) International Publication Number: **WO 00/37653** (43) International Publication Date: 29 June 2000 (29.06.00)

(21) International Application Number: PCT/US99/30707

(22) International Filing Date: 22 December 1999 (22.12.99)

(30) Priority Data:
60/113,204 22 December 1998 (22.12.98) US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application
US 60/113,204 (CON)
Filed on 22 December 1998 (22.12.98)

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: STRUCTURAL BASIS FOR PTERIN FUNCTION IN NITRIC OXIDE SYNTHASE

(57) Abstract

This invention describes methods and elucidates the three dimensional structure of nitric oxide synthase and its variants. Also described are methods of structural analysis to determine the binding of pterin to endothelial nitric oxide synthase and methods for screening and identifying small molecule modulators of endothelial nitric oxide synthase proteins and their variants. The invention also describes methods for identifying drugs that modulate nitric oxide synthase and its variants and are effective against diseased states in which NO signaling is defective or insufficient.

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STRUCTURAL BASIS FOR PTERIN FUNCTION IN NITRIC OXIDE SYNTHASE

The government owns rights in the present invention pursuant to grant numbers GM 32688, GM 52419 and HL 30050 from the National Institutes of Health.

1. Field of the Invention

The present invention relates generally to the fields of protein structure and crystallography. More particularly, it concerns the three dimensional structure of nitric oxide synthase.

2. Description of Related Art

Nitric oxide (NO°), a free radical, is an ubiquitous signaling molecule which participates in diverse cellular processes, including regulation of blood pressure, neurotransmission, and the immune response (Dinerman *et al.,* 1993).

NO, is well recognized as having various biologically relevant activities. For example, NO activates soluble guanylate cyclase in vascular smooth muscle cells which in turn increase cyclic guanosine monophosphate (cGMP) resulting in vasorelaxation, (Waldman *et al.,* 1987) and ultimately leads to vasodilation and a reduction in blood pressure. It is well established that the NOS family of enzymes form nitric oxide from L-arginine, and the NO produced is responsible for the endothelium dependent relaxation and activation of soluble guanylate cyclase, neurotransmission in the central and peripheral nervous systems, and activated macrophage cytotoxicity (Sessa, William C., 1994).

NO° production is tightly regulated by nitric oxide synthases (NOS), a family of enzymes of which three genetically encoded isoforms have been identified (Knowles and Moncada, 1994; Marietta, 1993; Masters *et al.,* 1996). The neuronal (nNOS) and endothelial NOS (eNOS) are constitutive with post-translational regulation of enzyme

activity. The inducible isoform (iNOS) is regulated mostly at the level of transcription. All three isoforms of NOS oxidize L-arginine to L-citrulline and NO.

Each NOS isoform consists of a heme domain linked via a calmodulin binding linker peptide to a P450 reductase-like diflavin domain giving a large polypeptide (130 kDa - 160 kDa). Only dimeric NOS is catalytically active. Upon Ca^{2+}/calmodulin binding, the FAD of the reductase domain transfers reducing equivalents from NADPH to FMN, which in turn, reduces the heme iron. Reduction of the heme iron leads to O_2 activation followed by oxidation of a L-Arg guanidino N atom to NO° and L-citrulline. All three NOS isoforms exhibit an absolute requirement for tetrahydrobiopterin, (referred to as BH_4), as a cofactor to function (Tayeh and Marietta, 1989; Kwon *et al.*, 1989), but the precise role it plays has remained elusive (Hemmens and Mayer, 1997).

Due to the linking of the heme and flavin domains, the various NOS isoforms are large and range in size from 130 to 160 kDa. The N-terminal domain of NOS contains the heme active center where L-arginine and BH_4 bind. While the flavin reductase domain is similar in sequence to the P450 reductase, the NOS heme domain bears little resemblance in sequence to P450s even though the NOS heme domain exhibits characteristics strikingly similar to those of cytochrome P450 monooxygenases.

Recently, the structures of mouse iNOS heme domains and cytochrome P450 reductase have both been determined (Crane and Trainer, 1997; Wang *et al.*, 1997). In both of these studies, short versions of the full length iNOS were used. For iNOS, the heme domain consisting of residues 115-498 was used, whereas for P450 reductase the soluble fragment (amino acids 57-676) was used. These investigations revealed that the flavin domain of P450 reductase is structurally similar to flavodoxins while the FAD-NADPH domain is structurally homologous to ferrodoxin reductase. Additionally, a linker domain connects the FMN and FAD domains. The flavins are only 4Å apart which blocks the edge of FMN that would normally be exposed in flavodoxins.

Despite the extensive characterization of the heme domain of iNOS, little is known about the structure of eNOS or the BH_4 binding domain therein. To understand the basis for the obligatory pterin cofactor requirement in catalysis and structure, there is a need to solve the structure of the eNOS heme domain, both in the presence and absence of BH_4. Such findings will have major implications in drug discovery and mechanisms of action of the proteins.

SUMMARY OF THE INVENTION

To overcome the limitations of the art the present inventors have developed methods for the structural analysis of endothelial nitric oxide synthase. In one embodiment the method comprises: a) subcloning a gene encoding the endothelial nitric oxide synthase in an expression vector; b) obtaining expression of the endothelial nitric oxide synthase protein from the vector; c) purifying the endothelial nitric oxide synthase protein; d) preparing an endothelial nitric oxide synthase protein sample amenable for crystallization; e) crystallizing the endothelial nitric oxide synthase protein sample; and f) performing a three-dimensional structural analysis of the endothelial nitric oxide synthase by x-ray crystallography.

In one embodiment, the endothelial nitric oxide synthase protein expressed is a variant endothelial nitric oxide synthase protein. In one aspect of the method, the crystallizing comprises practicing a sitting drop vapor-diffusion method. To compare the crystal structures in the presence and absence of tetrahydrobiopterin, in one embodiment, the crystallizing is performed in the presence of tetrahydrobiopterin while in another embodiment, the crystallizing is performed in the absence of tetrahydrobiopterin. In a further embodiment the crystal structures are determined by performing x-ray crystallography on the endothelial nitric oxide synthase protein crystallized both in the presence and in the absence of tetrahydrobiopterin.

The invention also describes a method of structural analysis to determine the binding of pterin to endothelial nitric oxide synthase that comprises: a) determining the

crystal structure of a dimeric heme domain of the endothelial nitric oxide synthase in the presence of the pterin; and b) determining the crystal structure of a dimeric heme domain of the endothelial nitric oxide synthase in the absence of the pterin; and comparing the crystal structures. In one embodiment the pterin is tetrahydrobiopterin. In a specific embodiment, the tetrahydobiopterin is (1'R,2'S,6R)-5,6,7,8-tetrahydrobiopterin. In one aspect, the crystal structure is determined at a resolution of between about 1.9 Å to about 2.1 Å.

The invention also describes methods for screening and identifying small molecule modulators of endothelial nitric oxide synthase proteins comprising: a) providing a pterin-free endothelial nitric oxide synthase structure; b) screening the small molecule modulators for their ability to bind to a pterin-binding site of the endothelial nitric oxide synthase ; and c) performing assays to determine the ability of the small molecule modulators to modulate the activity of endothelial nitric oxide synthase. A modulator is defined herein as a molecule that is capable of activating or inhibiting the activity of endothelial nitric oxide synthase. The molecule can be a small molecule.

In one embodiment, the small molecule modulator inhibits endothelial nitric oxide synthase. In another embodiment, the small molecule modulator activates endothelial nitric oxide synthase. In one embodiment, the endothelial nitric oxide synthase protein expressed is a variant endothelial nitric oxide synthase protein. In another embodiment, the pterin is tetrahydrobiopterin. In a specific embodiment, the tetrahydobiopterin is (1'R,2'S,6R)-5,6,7,8-tetrahydrobiopterin.

In one aspect of the method, the small molecule modulators are molecules and chemical-fragments from chemical-fragment libraries. In another aspect, the screening is performed by computerized methods. In yet another aspect of the method, the assays to determine the activity of endothelial nitric oxide synthase are performed *in vitro* or *in vivo*.

In another embodiment, the invention describes methods for identifying drugs against diseased states in which nitric oxide signaling is defective or insufficient comprising: a) providing a tetrahydrobiopterin-free endothelial nitric oxide synthase structure; b) screening the drugs for their ability to bind the tetrahydrobiopterin binding site; and c) performing assays to determine the ability of the drugs to activate the endothelial nitric oxide synthase. The diseased states include but are not limited to conditions wherein defective or insufficient nitric oxide signaling leads to impaired neurotransmission; impaired insulin release; impaired penile erection; impaired vasorelaxation; and impaired oxygen detection.

The invention also provides endothelial nitric oxide synthase structure, obtained by the process comprising: a) subcloning a gene encoding the endothelial nitric oxide synthase in an expression vector; b) obtaining expression of the endothelial nitric oxide synthase protein from the vector; c) purifying the endothelial nitric oxide synthase protein; d) preparing an endothelial nitric oxide synthase protein sample amenable for crystallization; e) crystallizing the endothelial nitric oxide synthase protein sample; and f) performing a three-dimensional structural analysis of the endothelial nitric oxide synthase by x-ray crystallography. The structure of the endothelial nitric oxide synthase is described in the specification.

The invention also provides methods for screening and identifying a candidate substance with the ability to inhibit endothelial nitric oxide synthase comprising: a) obtaining a cell with endothelial nitric oxide synthase activity; b) admixing the candidate substance with the cell; and c) determining the ability of the candidate substance to inhibit the endothelial nitric oxide synthase activity of the cell.

The invention further provides a method for screening and identifying a candidate substance with the ability to inhibit endothelial nitric oxide synthase comprising: a) obtaining a purified endothelial nitric oxide synthase; b) admixing the endothelial nitric oxide synthase with the candidate substance; and c) performing X-ray crystallography analysis to determine the binding of the candidate substance.

A" or "an" is defined herein to mean "at least one" when used in combination with the term "comprising" in the specification and claims.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. Panel A, schematic representation of the eNOS heme domain dimer viewed perpendicular to the dyed axis of symmetry. The two BH_4 molecules are shown as yellow space-filled models. The zinc located along the dyed axis of symmetry is highlighted as a red ball and labeled. Panel B, molecular surface map of the electrostatic potential of the eNOS heme domain dimer calculated using GRASP. The blue an99d red contours represent positive and negative potential, respectively. Fully saturated color indicates a potential of 5 kT. This view is rotated 90° from the orientation shown in Panel A such that the viewer is looking toward the ZnS_4 center directly along the two-fold relating the monomers. The surface surrounding the ZnS_4 is the most extensive electropositive region on the dimer and could provide an electrostatic docking site for the FMN/FAD reductase. This could enable the electron donors (FMN domains of the reductase) to approach close to the ZnS_4 center thereby providing a conduit to

either/both pterin and heme groups. Interestingly, both Mss4 and LIM proteins utilize ZnS_4 containing surfaces to mediate protein-protein interaction (Yu and Schreiber, 1995; Rabbits and Boehm, 1990; Schmeichel and Beckerle, 1994).

FIG. 2. Stereo view of the 2Fo-Fc 1.9Å omit electron density map around the ZnS_4 center. The map was obtained from model phases after a round of simulated annealing with the atoms shown excluded from the refinement. The map is contoured at 1σ (blue) and 10σ (black). The zinc ion was identified using anomalous dispersion effects characteristics for the metal. X-ray wavelengths of 1.280 Å and 1.286 Å (zinc absorption edge, λ = 1.283 Å) were chosen using a tunable synchrotron X-ray source. Zinc exhibits significant anomalous scattering effects at 1.280 Å with little anomalous scattering contribution at 1.286 Å. Heme Fe exhibits some anomalous scattering at both wavelengths (iron absorption edge, λ = 1.739 Å). Direct methods (Sheldrick, 1997) were also used to independently confirm the location of the metal center. The cysteine residues of eNOS involved in zinc coordination are strictly conserved in all NOS sequences known to date indicating that the metal center is a common feature in all NOS isoforms. The ligands, Cys 96 and Cys 101, are part of a small 3-stranded antiparallel β-sheet (2 strands from one monomer and 1 strand from the other) that orients Cys 96 and Cys 101 in the same direction directly across antiparallel strands. In addition to β-strand main chain H-bonds, Sγ (96) and Sγ (101) form H-bonds with the peptide NH of residues 102 and 103, respectively. Crane *et al.* (1998) interpreted this region in iNOS as an inter-subunit disulfide bond between symmetry related Cys 109 residues which corresponds to one of the ligands in eNOS, Cys 101. The reason for the discrepancy is not clear. However, the iNOS heme domain dimer structure was solved at medium resolution (2.6 Å) and Crane *et al.* (1998) noted a disordering in residues 101-107 immediately preceding Cys 109. Considering the strongly reducing conditions in the cytosol, the formation of a disulfide would be both kinetically and thermodynamically disfavored (Braakman *et al.*, 1994). Hence, the inventors conclude that the loss of zinc in the iNOS structure led to the disordering of the polypeptide chain. ZnS_4 centers have been observed in four other enzymes where they play a structural role (Lipscomb and Sträter, 1996; Tsukihara *et al.*, 1995; Vallee and Auld, 1993). In *E. coli* Ada protein a

catalytic function of ZnS_4 has been demonstrated (Lipscomb and Sträter, 1996; Tsukihara *et al.*, 1995; Vallee and Auld, 1993).

FIG. 3. Comparison of the pterin-free and -bound structures at BH_4 binding site. L-Arg was found in the BH_4 binding pocket in one subunit whereas only glycerol and water molecules could be modeled in the other subunit. Panels A and B are the 2Fo-Fc omit electron density maps contoured at 1σ with arginine or glycerol excluded in the calculation, respectively. Side chains of the same color belong to the same monomer. Panel C shows BH_4 being sandwiched between Trp 449 of the same subunit (green) and Phe 462 from the other (cyan). Among the extensive H-bonding interactions of BH_4 to protein, two crucial ones are between N at position 3 of the pterin ring and a heme propionate, and between the OH in dihydroxypropyl side chain of BH_4 and Ser104 carbonyl oxygen. These interactions are closely mimicked by L-arginine with its guanidino nitrogen and primary amino group, respectively, as depicted in pang D. In addition, a new water molecule in panel D satisfies the H-bonding interactions of the BH_4 amino group. Therefore, L-Arg in the BH_4 site is able to closely mimic the H-bonding and aromatic stacking interactions in the BH_4 complex. The structure of the BH_4 complex in panel C has L-Arg bound in the heme pocket while in panel D, the-inhibitor, SEITU, is in the heme pocket. The protein structure of the inhibitor and substrate complexes are essentially identical. In addition, the structure of the substrate or inhibitor complex with BH_4 bound exhibit no differences at the BH_4 site.

FIG. 4. The ZnS_4 center and its relation to BH_4. The metal ion (white ball) is equidistant from each BH_4, a distance of 12 Å. The peptide carbonyl oxygen of Ser 104 H-bonds with one BH_4 OH group. ZnS_4 plays an effector role in helping to form and stabilize the pterin binding pocket which, in turn, promotes substrate binding. Therefore, the ZnS_4 center, BH_4, and substrate are all structurally linked at the dimer interface. Both the zinc atom and its cysteine ligands are accessible to solvent.

FIG. 5. The proposed mechanism for pterin radical formation in NOS catalysis. I-BH_4 showing the ring numbering scheme. The pKa of N3 is near 10.6. II-

BH_4 mono cation form and III the $BH_4^{+\bullet}$ radical, respectively. The inventors are proposing that NOS is designed to stabilize III, the radical cation. Stabilization of aromatic cation radicals is reminiscent of cytochrome c peroxidase which is designed to stabilize a cationic Trp radical essential for catalysis (Sivaraja *et al.*, 1989; Houseman *et al.*, 1993).

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

NO is a key intracellular signal and defensive cytotoxin in the nervous, muscular, cardiovascular, and immune systems (Moncada and Higgs, 1993; Schmidt and Walter, 1994; Nathan, 1997; Christopherson and Bredt, 1997; Marletta, 1993; Mayer and Werner, 1995; Masters *et al.*, 1996; Steuhr *et al.*, 1997). eNOS produces low NO concentrations for neurotransmission, insulin release, penile erection, vasorelaxation, oxygen detection, and the like. Since NO was voted the molecule of the year by *Science* in 1992, there has been a tremendous amount of work on the pharmacological properties of this molecule. Despite all these studies, little is known about the structure of eNOS. Once the structural determination of eNOS has been made, designing agents to potentiate or inhibit the action of NO becomes a realizable goal. The present invention is directed to addressing these needs.

A. The Present Invention

The present invention describes the crystal structure of the dimeric heme domain of endothelial nitric oxide synthase (eNOS). This structure was determined both in the presence and absence of (1'R,2'S,6R)-5,6,7,8-tetrahydrobiopterin (BH_4) to understand its role as an indispensable cofactor in nitric oxide biosynthesis. The BH_4 bound structure at 1.9 Å reveals a novel zinc tetrathiolate (ZnS_4) sandwiched at the dimer interface which functions by maintaining the integrity of the pterin and substrate binding sites. The pterin-free structure at 2.1 Å unambiguously establishes an obligatory function for BH_4 in catalysis and rules out a role in the dimerization process. These structures suggest a reaction mechanism that involves a pterin radical. The unusual finding that an L-arginine (L-Arg) is bound at the BH_4 site in the pterin-free structure suggests evolution of cofactor

recognition from a common L-Arg-binding ancestor in the primordial NOS catalytic machinery.

Here the inventors report the 1.9 Å crystal structure of the dimeric heme domain of constitutive eNOS (Table 1), which maintains the catalytic site for $NO^{\bullet}$ synthesis. The overall fold of the bovine eNOS heme domain (FIG. 1) is similar to that reported for the 2.6 Å structure of the iNOS heme domain dimer (Crane *et al.*, 1998). eNOS belongs to the α/β protein class and the quaternary structure is characterized by a tightly packed dimer interface which buries 3000 $Å^2$ per subunit of solvent accessible surface (55% hydrophobic and 45% polar).

To understand the basis for the obligatory pterin cofactor requirement in catalysis and structure, the inventors have solved the structure of eNOS heme domain both in the presence and absence of BH_4 resulting in three major structural findings. First, the inventors have found a novel ZnS_4 center (in both pterin-bound and -free structures) located at the bottom of the dimer interface (FIG. 1) with the metal tetrahedrally coordinated by two pairs of symmetry-related cysteine residues (FIG. 2) from each subunit. BH_4 H-bonds directly with a heme propionate which also entertains H-bonds with the substrate, L-Arg (FIG. 3C). The zinc is positioned equidistant from each heme (21.6 Å) with one of its ligands, Cys 101, separated by only two residues from Ser 104 which H-bonds directly to BH_4 (FIG. 4). In addition, Val 105 forms a direct nonbonded contact with BH_4. Therefore, disruption of the metal center either by demetallation or ligand removal via mutagenesis will distort this region of the polypeptide chain resulting in greatly diminished affinity for BH_4. Since BH_4 couples directly to the heme, alterations at the pterin site will in turn affect the heme pocket and L-Arg binding. A number of studies (Chen *et al.*, 1995; Rodríguez-Crespo *et al.*, 1997; Ghosh *et al.*, 1997; Venema *et al.*, 1997; Miller *et al.*, 1997) confirm the dramatic loss in protein stability, catalytic activity, and BH_4 binding upon removal of Cys 96 and/or Cys 101 in eNOS or their counterparts in the inducible and neuronal isoforms.

The identification of the new ZnS_4 center in NOS unambiguously establishes the structural role played by these cysteine residues in forming and maintaining the integrity of the pterin site. The inventors' finding of zinc in eNOS has pathophysiological implications as well. Inherited vascular dysfunction may arise from mutations that specifically weaken zinc affinity resulting in a dysfunctional eNOS. For example, in familial amyotropic lateral sclerosis (ALS), over 50 independent mutations in Cu/Zn-superoxide dismutase give rise to a common toxic phenotype invariably characterized by decreased zinc affinity up to 100,000 fold (Lyons *et al.*, 1996; Crow *et al.*, 1997).

Second, in the absence of BH_4, the overall eNOS heme domain structure remains unchanged both in the tertiary topology and quaternary structure. These results sharply contrast with the conclusions of Crane *et al.*, (1998) who proposed that large conformational changes concomitant with BH_4 binding were necessary for inducible NOS dimerization. By comparison of the pterin-bound and pterin-free structures, it is clear that the site preexists (FIG. 3) and does not form via an induced fit process. The pterin-free structure also does not show any major changes at the active site either in the substrate- or inhibitor (*S*-ethylisothiourea, SEITU)-bound conformations.

Third, in addition to the new ZnS_4 center, the most striking and novel finding is the identification of an L-Arg bound to the pterin site in the absence of BH_4 (FIG. 3A and FIG. 3D). The mode of L-Arg interaction at the pterin site surprisingly mimics that entertained by BH_4 itself. Two crucial H-bonds, one between a guanidino N with the heme propionate and the other between the primary amino group and Ser 104 are strictly conserved (FIG. 3D). Moreover, the planar guanidinium group is sandwiched between two aromatic groups, one from each monomer, exactly as in the BH_4 complex. Solvent interactions also are similar to the BH_4 complex. The affinity for L-Arg must be great since no exogenous L-Arg was added during crystallization. The specificity of L-Arg recognition at the pterin site appears to require both the guanidino function and the primary amino group since SEITU was unable to displace L-Arg from the pterin site.

The inventors' observation involving L-Arg mimicry of BH_4 has not been previously observed and has broad evolutionary implications. It is possible that the pterin site was originally an L-Arg binding site and later evolved into a BH_4 site. The "arginine paradox" (McDonald *et al.*, 1997), which refers to the ability of extracellular L-Arg to drive $NO^{\bullet}$ biosynthesis, amidst the large intracellular substrate pool, suggests limited substrate availability in specialized cellular compartments and may have favored the evolution of a second L-Arg site as a substrate reserve in a primordial setting. The inventors propose that the higher affinity of NOS for BH_4 (K_d ~ 20 nM; Werner-Felmayer and Gross, 1996) evolved to repel competition from L-Arg which is abundant in cells (0.2 - 0.8 mM; Hecker *et al.*, 1990). A striking corollary can be established between the inventors' finding and the ability of *Tetrahymena* group I catalytic RNA to specifically recognize L-Arg as a mimic for guanosine binding (Yarus, 1988).

Given this elegant mimicry of the obligatory cofactor by L-Arg, the question arises as to why NOS chose a pterin in place of L-Arg for sustaining function. The strict requirement for reduced pterin cannot apparently be explained by a purely structural role since L-Arg can serve this same function. BH_4 likely plays a direct functional role. A well-known function of pterin is to cycle between quinonoid BH_2 and BH_4 in metal-dependent aromatic amino acid hydroxylases (Kaufman, 1997). To date, direct evidence for both pterin cycling and pterin function in NOS is lacking (Hemmens and Mayer, 1997). The inventors' finding that the pterin site in NOS recruits L-Arg, provides structural insights on why BH_4 can serve as a single electron donor. The guanidino group of L-Arg is one of the strongest organic bases and the ability of the pterin site to bind L-Arg argues in favor of preferential binding of a fully protonated species of BH_4. Owing to the essentially identical nature of the pterin site in both the L-Arg and BH_4 complexes, it appears that bound BH_4 experiences the same electrostatic environment as L-Arg.

The inventors propose a mechanism (FIG. 5) in which the NOS pterin site modulates the pKa of N5 of the pyrazine ring and also provides an "acidic" milieu known to stabilize pterin radicals (Pfleiderer, 1985; Kappock and Caradonna, 1996; Eberlein *et*

al., 1984) that can serve as one electron donors (Bec *et al.*, 1998) in $NO^{\bullet}$ biosynthesis. Cycling from the pterin radical back to BH_4 may be achieved *via* electron transfer from the reductase domain while the pterin remains bound to NOS. Another important structural feature that will substantially contribute to pterin radical stabilization is the π stacking interaction with Trp 449. Such aromatic stacking is not found in binding sites of other pterin utilizing enzymes (Bourne *et al.*, 1991; Auerbach *et al.*, 1997), but resonance stabilization of flavin semiquinone radical found in flavoproteins are mediated through stacking interactions (Massey, 1994; Wang *et al.*, 1997). NOS provides an interesting scenario in which an enzyme has evolved to produce a pterin function that may mimic flavoprotein systems in structure and function.

Finally, the pterin-free eNOS structure also has relevance to human medicine. There is strong evidence for superoxide generation by the heme domain of eNOS in the absence of BH_4 thereby leading to potential pathophysiology. Endothelial dysfunction is reversed in hypercholesterolemic patients treated with BH_4 and has been shown to be a NOS related action (Stroes *et al.*, 1997; Piper, 1997; Kinoshita *et al.*, 1997; Cosentino *et al.*, 1998). It is conceivable that BH_4 deficient eNOS may be a reality in vascular pathologies in which L-Arg could substitute at the BH_4 site in pterin-depleted states. The availability of pterin-free eNOS structure paves the way for rational design of both pterin-dependent and independent activators that can restore endothelial function. These and other implications of the findings present herein are discussed in further detail herein below.

B. Crystallization Techniques

The techniques used for the crystallization of a protein for crystallographic resolution of protein are well known to those of skill in the art. One technique for crystallization is referred to as the microbatch technique. The microbatch technique is ideal for the rapid determination of the phase diagram of a protein. If the concentration of crystallizable protein is plotted against the concentration of a precipitant, microbatch results can be used to divide the space represented into several areas. Microbatching has

been extensively described in the literature, see for example, Chayen *et al.*, 1990; Chayen *et al.*, 1992; Chayen *et al.*, 1994.

In microbatching, at high concentrations of both protein and precipitant, the protein precipitates as an amorphous material. At lower concentrations, crystal nuclei may form, which may grow to form diffracting crystals. At still lower concentrations, nuclei will not form, so generally no crystals appear. However, if a nucleus or crystal is placed in such a solution, it will grow to form a large crystal. This area, where crystal growth but not nucleation takes place, is sometimes referred to as "the metastable zone". At the lowest concentrations, the protein is completely soluble. It is often found that crystals grown in the metastable zone are better ordered and diffract better than crystals grown at higher concentrations. The microseeding approach described herein below includes a simple method of finding the metastable zone and introducing crystal seeds to it.

Single crystals of a given protein are generally obtained by microseeding, a valuable measurement in this step is the precipitation point of a protein at a single protein concentration which is described by Stewart and Khimasia (1994). A well with a well-formed crystal is selected and the crystal is transferred to a glass depression plate containing 40 µl of harvesting buffer with a high PEG concentration. The initial crystal is ground up with a needle or a glass fibre with a ball at the tip. The resulting suspension is added to an Eppendorf tube containing 100 µl of the harvesting buffer the tube was centrifuged for a five minutes at around 100 g. The supernatant from this step contains the seeds; this supernatant may then be diluted to yield varying concentrations of seeding solution. The seeding solutions are used to seed sitting drop crystallization trials in appropriate crystallization plates (e.g., CrystalClear plates Douglas Instruments). 100 µl of solution was used in the reservoirs. Next, buffer, protein and PEG are dispensed automatically into the sample wells of the CrystalClear plates. By dispensing droplets marginally below the reservoir concentration, the need for equilibration before seeding may be avoided - the concentration was not so low that the nuclei dissolved. Finally 0.3 µl of each of the seeding solutions produced is added by hand to each sample well with a

5 μl Hamilton syringe. The plates were then sealed and crystals allowed to form. Small single crystals will appear after several days.

Large crystals are obtained by macroseeding. Using a rayon loop, a small single crystal is transferred into reservoir solution, allowed to wash for several minutes, and then transferred into another drop that has been equilibrated for 3-5 days. The same reservoir and drop condition used to obtain the initial aggregates also are used for the subsequent micro and macroseeding. The crystals attain their maximum size in 5-10 days following macroseeding. Typical crystal dimensions are 0.3 mm × 0.3 mm × 0.6 mm.

C. Specific eNOS Active Site Modifications

Given that the present invention has determined the crystal structure of eNOS, it is now possible to modify various specific residues within the protein to determine the roles of particular residues within the active site. The following is a discussion based upon changing the amino acids of a protein to create an equivalent, or even an improved, second-generation molecule. The amino acid changes may be achieved by changing the codons of the DNA sequence, according to the following codon table, Table 1:

TABLE 1

Amino Acid Names and abbreviations			Codons					
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Cysteine	Cys	C	UGC	UGU				
Aspartic acid	Asp	D	GAC	GAU				
Glutamic acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

It is known that certain amino acids may be substituted for other amino acids in a protein structure in order to modify or improve its antigenicity or activity (see, *e.g.*, Kyte and Doolittle, 1982; Hopp, U.S. Patent 4,554,101, incorporated herein by reference). For example, through the substitution of alternative amino acids, small conformational changes may be conferred upon a polypeptide which result in increased activity or stability. Alternatively, amino acid substitutions in certain polypeptides may be utilized to provide residues which may then be linked to other molecules to provide peptide-molecule conjugates which retain enough antigenicity of the starting peptide to be useful for other purposes. For example, a selected eNOS peptide bound to a solid support might be constructed which would have particular advantages in diagnostic embodiments.

The importance of the hydropathic index of amino acids in conferring interactive biological function on a protein has been discussed generally by Kyte and Doolittle (1982), wherein it is found that certain amino acids may be substituted for other amino acids having a similar hydropathic index or core and still retain a similar biological activity. As displayed in Table 2 below, amino acids are assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics. It is believed that the relative hydropathic character of the amino acid determines the secondary structure of the resultant protein, which in turn defines the interaction of the protein with substrate molecules. Preferred substitutions which result in an antigenically equivalent peptide or protein will generally involve amino acids having index scores within ±2 units of one another, and more preferably within ±1 unit, and even more preferably, within ±0.5 units.

TABLE 2

Amino Acid	Hydropathic Index
Isoleucine	4.5
Valine	4.2
Leucine	3.8
Phenylalanine	2.8
Cysteine/cystine	2.5
Methionine	1.9
Alanine	1.8
Glycine	-0.4
Threonine	-0.7
Tryptophan	-0.9
Serine	-0.8
Tyrosine	-1.3
Proline	-1.6
Histidine	-3.2
Glutamic Acid	-3.5
Glutamine	-3.5
Aspartic Acid	-3.5
Asparagine	-3.5
Lysine	-3.9
Arginine	-4.5

Thus, for example, isoleucine, which has a hydropathic index of +4.5, will preferably be exchanged with an amino acid such as valine (+ 4.2) or leucine (+ 3.8). Alternatively, at the other end of the scale, lysine (- 3.9) will preferably be substituted for arginine (-4.5), and so on.

Substitution of like amino acids may also be made on the basis of hydrophilicity, particularly where the biological functional equivalent protein or peptide thereby created is intended for use in immunological embodiments. U.S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with its

immunogenicity and antigenicity, *i.e.* with an important biological property of the protein.

As detailed in U.S. Patent 4,554,101, each amino acid has also been assigned a hydrophilicity value. These values are detailed below in Table 3.

TABLE 3

Amino Acid	Hydrophilic Index
arginine	+3.0
lysine	+3.0
aspartate	+3.0 ± 1
glutamate	+3.0 ± 1
serine	+0.3
asparagine	+0.2
glutamine	+0.2
glycine	0
threonine	-0.4
alanine	-0.5
histidine	-0.5
proline	-0.5 ± 1
cysteine	-1.0
methionine	-1.3
valine	-1.5
leucine	-1.8
isoleucine	-1.8
tyrosine	-2.3
phenylalanine	-2.5
tryptophan	-3.4

It is understood that one amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

Accordingly, these amino acid substitutions are generally based on the relative similarity of R-group substituents, for example, in terms of size, electrophilic character, charge, and the like. In general, preferred substitutions which take various of the foregoing characteristics into consideration will be known to those of skill in the art and include, for example, the following combinations: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

As outlined above, amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions which take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine (See Table 4, below). The present invention thus contemplates functional or biological equivalents of an eNOS or variant eNOS polypeptide as set forth above.

TABLE 4

Original Residue	Exemplary Substitutions
Ala	Gly; Ser
Arg	Lys
Asn	Gln; His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Ala
His	Asn; Gln
Ile	Leu; Val
Leu	Ile; Val
Lys	Arg
Met	Met; Leu; Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp; Phe
Val	Ile; Leu

Biological or functional equivalents of a polypeptide can also be prepared using site-specific mutagenesis. Site-specific mutagenesis is a technique useful in the preparation of second generation polypeptides, or biologically functional equivalent polypeptides or peptides, derived from the sequences thereof, through specific mutagenesis of the underlying DNA. As noted above, such changes can be desirable where amino acid substitutions are desirable. The technique further provides a ready ability to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides

of the deletion junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art, as exemplified by Adelman, *et al.* (1983). As will be appreciated, the technique typically employs a phage vector which can exist in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage (Messing, *et al.*, 1981). These phage are commercially available and their use is generally known to those of skill in the art.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector which includes within its sequence a DNA sequence which encodes all or a portion of the eNOS or variant eNOS enzyme polypeptide sequence selected. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically, for example, by the method of Crea *et al.*, (1978). This primer is then annealed to the singled-stranded vector, and extended by the use of enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells such as *E. coli* cells and clones are selected which include recombinant vectors bearing the mutation. Commercially available kits come with all the reagents necessary, except the oligonucleotide primers.

In addition, peptides derived from these polypeptides, including peptides of at least about 6 consecutive amino acids from these sequences, are contemplated. Alternatively, such peptides may comprise about 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59 or 60 consecutive residues. For example, a peptide that comprises 6 consecutive amino acid residues may

comprise residues 1 to 6, 2 to 7, 3 to 8 and so on of the eNOS protein. Such peptides may be represented by the formula

x to (x + n) = 5' to 3' the positions of the first and last consecutive residues

where x is equal to any number from 1 to the full length of the eNOS protein and n is equal to the length of the peptide minus 1. Where the peptide is 10 residues long (n = 10-1), the formula represents every 10-mer possible for each antigen. For example, where x is equal to 1 the peptide would comprise residues 1 to (1 + [10-1]), or 1 to 10. Where x is equal to 2, the peptide would comprise residues 2 to (2 + [10-2]), or 2 to 11, and so on.

Syntheses of peptides are readily achieved using conventional synthetic techniques such as the solid phase method (*e.g.*, through the use of a commercially available peptide synthesizer such as an Applied Biosystems Model 430A Peptide Synthesizer). Peptides synthesized in this manner may then be aliquoted in predetermined amounts and stored in conventional manners, such as in aqueous solutions or, even more preferably, in a powder or lyophilized state pending use.

In general, due to the relative stability of peptides, they may be readily stored in aqueous solutions for fairly long periods of time if desired, *e.g.*, up to six months or more, in virtually any aqueous solution without appreciable degradation or loss of antigenic activity. However, where extended aqueous storage is contemplated it will generally be desirable to include agents including buffers such as Tris or phosphate buffers to maintain a pH of 7.0 to 7.5. Moreover, it may be desirable to include agents which will inhibit microbial growth, such as sodium azide or Merthiolate. For extended storage in an aqueous state it will be desirable to store the solutions at 4°C, or more preferably, frozen. Of course, where the peptide(s) are stored in a lyophilized or powdered state, they may be stored virtually indefinitely, *e.g.*, in metered aliquots that may be rehydrated with a predetermined amount of water (preferably distilled, deionized) or buffer prior to use.

Of particular interest are peptides that represent antigenic epitopes that lie within the eNOS polypeptides of the present invention. An "epitope" is a region of a molecule that stimulates a response from a T-cell or B-cell, and hence, elicits an immune response from these cells. An epitopic core sequence, as used herein, is a relatively short stretch of amino acids that is structurally "complementary" to, and therefore will bind to, binding sites on antibodies or T-cell receptors. It will be understood that, in the context of the present disclosure, the term "complementary" refers to amino acids or peptides that exhibit an attractive force towards each other. Thus, certain epitopic core sequences of the present invention may be operationally defined in terms of their ability to compete with or perhaps displace the binding of the corresponding eNOS antigen to the corresponding eNOS-directed antisera.

The identification of epitopic core sequences is known to those of skill in the art. For example U.S. Patent 4,554,101 teaches identification and preparation of epitopes from amino acid sequences on the basis of hydrophilicity, and by Chou-Fasman analyses. Numerous computer programs are available for use in predicting antigenic portions of proteins, examples of which include those programs based upon Jameson-Wolf analyses (Jameson and Wolf, 1988; Wolf *et al.,* 1988), the program PepPlot® (Brutlag *et al.,* 1990; Weinberger *et al.,* 1985), and other new programs for protein tertiary structure prediction (Fetrow and Bryant, 1993) that can be used in conjunction with computerized peptide sequence analysis programs.

In general, the size of the polypeptide antigen is not believed to be particularly crucial, so long as it is at least large enough to carry the identified core sequence or sequences. The smallest useful core sequence anticipated by the present disclosure would be on the order of about 6 amino acids in length. Thus, this size will generally correspond to the smallest peptide antigens prepared in accordance with the invention. However, the size of the antigen may be larger where desired, so long as it contains a basic epitopic core sequence.

D. **Small Molecule Modulators of eNOS**

The present invention provides methods for screening and identifying small molecule modulators of eNOS proteins and identifies such compounds. One rationale behind the design of the small molecule eNOS protein modulators is that in the absence of BH_4 it is seen that the heme domain of eNOS generates superoxide radicals thereby leading to pathophysiology. For example, further endothelial dysfunction in hypercholesteremia is reversed as a result of BH_4 treatment. The activated eNOS protein is thus able to produce NO and restore endothelial function. The present invention provides a pterin-free eNOS structure that can be used to model drugs (i.e., ligands) that will ameliorate the effect of BH_4 depletion. Such ligands will be useful in any diseased state in which NO signaling is defective or insufficient.

The findings of the present invention will be exploited to design chemical ligands that bind to the active site of the different variant proteins to yield complexes with sufficient thermodynamic stability to effectively modulate the functional activity of the protein. To obtain appropriate ligands that bind to the active sites of different eNOS variant proteins, the inventors may utilize the technique of force-field docking of chemical fragments from both commercially available chemical fragment libraries, as well as in-house generated libraries, into the active electrophile-binding (H-) site in the derived crystal structure of each variant protein. The docked fragments will be energy-minimized and the binding energies computed and used to select candidate ligands.

Generation of eNOS modulators: Generation of modulators is accomplished by a rational drug development strategy involving force field docking and energy-minimization of chemical fragments and compounds into the active site of the eNOS protein(s). The. Additional chemical libraries also may be generated as necessary. The active compounds and chemical fragments can be drawn from chemical fragment libraries, such as that available in the Leapfrog database site and other structural components of the eNOS proteins will be derived from the crystal structure of the eNOS described by the present invention.

One potential substitution that confers a functional change to the eNOS protein is to replace Cys 101 and/or Ser 104 which bond to BH_4 (FIG. 4). In addition, Val 105, which forms a direct nonbonded contact with BH_4 may be altered. Therefore, disruption of the metal center either by demetallation or ligand removal via mutagenesis will distort this region of the polypeptide chain resulting in greatly diminished affinity for BH_4. Additional mutations are contemplated which may result in increased stability. For example, increased protein stability results from the addition of disulfide bonds and the creation of more hydrophobic interactions within the protein structure.

Based on the resultant DDH values obtained after energy minimization of chemical fragments/compounds, candidate modulators are selected and/or newly constructed from chemical fragments for synthesis and further analyses for their inhibitory or other action on the eNOS proteins. Selection criteria for such modulators for synthesis and further analysis includes lipophilicity, chemical stability, and availability or ease of synthesis.

If the identified and/or newly constructed potential inhibitors are not commercially available, then they will be synthesized using standard organic synthetic methodology, including heterocyclic ring construction and functionalization, and electrophilic and nucleophilic substitution reactions. Reaction mixtures will be separated by thin layer, flash silica gel column, and high performance liquid chromatography (TLC, CC and HPLC). The compounds will be purified using standard techniques modified as necessary. Characterization of synthetic products will be done by melting point determination, Fourier transform infrared (FT-1R), ultraviolet (UV), and high resolution nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry. Compounds for biological testing will be purified by preparative HPLC. The purity of compounds will be determined by elemental analysis and HPLC.

Candidate modulators of the present invention will be useful in the treatment of nitric oxide synthase mediated diseases and disorders, including neurodegenerative disorders, disorders of gastrointestinal motility and inflammation. These disease and

disorders include hypotension, septic shock, toxic shock syndrome, hemodialysis, IL-2 therapy such as in cancer patients, cachexia, immnunosuppression such as in transplant therapy, autoimmune and/or inflammatory indications including sunburn or psoriasis and respiratory conditions such as bronchitis, asthma, and acute respiratory distress (ARDS), myocarditis, heart failure, atherosclerosis, arthritis, rheumatoid arthritis, chronic or inflammatory bowel disease, ulcerative colitis, Crohn's disease, systemic lupus erythematosis (SLE), ocular conditions such as ocular hypertension and uveitis, type 1 diabetes, insulin-dependent diabetes mellitus, and cystic fibrosis. These compounds will be similar to those already described in the art in for example, U.S. Patent 5,821,261; U.S. Patent 5,821,267; U.S. Patent 5,807,886; U.S. Patent 5,776,979; U.S. Patent 5,767,160; U.S. Patent 5,728,728; U.S. Patent 5,723,451; U.S. Patent 5,710,181; U.S. Patent 5,688,499; U.S. Patent 5,684,008; U.S. Patent 5,674,907; U.S. Patent 5,645,839; U.S. Patent 5,629,322; U.S. Patent 5,585,402; U.S. Patent 5,543,430; U.S. Patent 5,480,999; U.S. Patent 5,436,271; U.S. Patent 5,380,945; U.S. Patent 5,362,747; U.S. Patent 5,296,466 and U.S. Patent 5,266,594 (each incorporated herein by reference). The compositions disclosed in these patents may be used as starting materials for rational drug design to yield modulators that best fit the crystal structure of NOS described herein.

The different compounds may have varying substituents which result in significant changes in binding energies of the compounds in the active site pocket of the eNOS protein. An individual skilled in the art of organic synthesis in light of the present disclosure will be able to prepare or identify a large variety of candidate molecules which would be expected to have eNOS modulatory effects in the light of the present disclosure.

The modulators identified may be inhibitors or stimulators of eNOS activity. Inhibitors will be used in treating various conditions where there is an advantage in inhibiting nitric oxide biosynthesis, as described in e.g., U.S. Patent 5,821,261; U.S. Patent 5,821,267; 5,807,886; U.S. Patent 5,789,442; U.S. Patent 5,789,395; U.S. Patent 5,776,979; U.S. Patent 5,756,540; 5,741,815; U.S. Patent 5,723,451; U.S. Patent 5,721,278; U.S. Patent 5,710; U.S. Patent 5,710,181; U.S. Patent 5,695,761; U.S. Patent

5,684,008; U.S. Patent 5,674,907; 5,645,839; U.S. Patent 5,629,322; U.S. Patent 5,585,402. Inhibition of NOS activity will be useful in treating conditions such as hypotension, inhibition of ovulation, inflammatory bowel disease, inflammation, autoimmune diseases and septic shock variety of cardiovascular fibrotic pathologies, such as that associated with left ventricular hypertrophy secondary to hypertension, myocardial infarction, myocarditis, and the like. Stimulators will be useful in treating conditions where there is an advantage to stimulating nitric oxide biosynthesis. Such conditions include diseases related to vasoconstriction, wherein the vasoconstriction is relieved by stimulating the NOS to produce native nitric oxide, e.g., as described in U.S. Patent 5,767,160; 5,543,430; stimulation of ovulation as described in U.S. Patent 5,721,278. NOS stimulators also are used to slow and reverse the process of fibrosis in the body, useful in the treatment of a variety of cardiovascular fibrotic pathologies, such as that associated with left ventricular hypertrophy secondary to hypertension, myocardial infarction, and myocarditis as described in U.S. Patent 5,645,839.

Screening for modulators of eNOS: Within certain embodiments of the invention, methods are provided for screening for modulators of eNOS protein activity. Such methods may use labeled eNOS proteins or analogs, anti-eNOS proteins or anti-eNOS antibodies and the like as reagents to screen small molecule and peptide libraries to identify modulators of eNOS protein activity. Within one example, a modulator screening assay is performed in which cells expressing eNOS proteins are exposed to a test substance under suitable conditions and for a time sufficient to permit the agent to effect activity of eNOS proteins.

Generally the test substance is added in the form of a purified agent. However, it is also contemplated that test substances useful within the invention may include substances present throughout the handling of test sample components. For example, host cell factors that are present in a cell lysate may be used for generating the test sample. Such endogenous factors may be segregated between the test and control samples, for example, by using different cell types for preparing lysates. In such

preparations, the cell type used for preparing the test sample expresses a putative test substance that is not expressed by the cell type used in preparing the control sample.

The active compounds may include fragments or parts of naturally-occurring compounds or may be only found as active combinations of known compounds which are otherwise inactive. However, prior to testing of such compounds in humans or animal models, it may be necessary to test a variety of candidates to determine which have potential.

Accordingly, in screening assays to identify agents which alter the activity of eNOS proteins, it is proposed that compounds isolated from natural sources, such as animals, bacteria, fungi, plant sources, including leaves and bark, and marine samples may be assayed as candidates for the presence of potentially useful pharmaceutical agents. It will be understood that the pharmaceutical agents to be screened could also be derived or synthesized from chemical compositions or man-made compounds.

In these embodiments, the present invention is directed to a method for determining the ability of a candidate substance to decrease the eNOS activity of cells, the method including generally the steps of:

(a) obtaining a cell with eNOS activity;
(b) admixing a candidate substance with the cell; and
(c) determining the ability of the candidate substance to inhibit the eNOS activity of the cell.

To identify a candidate substance as being capable of decreasing eNOS activity, one would measure or determine the basal eNOS status of the cell prior to any additions or manipulation. One would then add the candidate substance to the cell and re-determine the eNOS activity in the presence of the candidate substance. A candidate substance which decreases the eNOS activity relative to the composition in its absence is

indicative of a candidate substance being an inhibitor of eNOS. A similar assay may be set up to determine whether the candidate substance is a stimulator of eNOS activity.

"Effective amounts", in certain circumstances, are those amounts effective to reproducibly alter eNOS activity in an assay in comparison to their normal levels. Compounds that achieve significant appropriate changes in activity will be used. If desired, a battery of compounds may be screened *in vitro* to identify other agents for use in the present invention.

A significant change in eNOS activity is represented by a change in eNOS protein activity levels of at least about 30%-40%, and most preferably, by a change of at least about 50%, with higher values of course being possible. Assays that measure eNOS activity in cells are well known in the art and may be conducted *in vitro* or *in vivo*, and have been described elsewhere in the specification.

Quantitative *in vitro* testing of the eNOS modulators is not a requirement of the invention as it is generally envisioned that the agents will often be selected on the basis of their known properties or by structural and/or functional comparison to those agents already demonstrated to be effective. Therefore, the effective amounts often will be those amounts proposed to be safe for administration to animals in another context.

E. Antibodies to eNOS

Within certain embodiments of the present invention, antibodies raised against eNOS may be useful in aiding the identification of drugs. An antibody that recognizes the active site of an enzyme will act as a mimic of the drug that fits that active site. Using this information, drugs may be designed that mimic the shape of such an antibody.

Antibodies to eNOS variant peptides or polypeptides may be readily prepared through use of well-known techniques, such as those exemplified in U.S. Patent 4,196,265. Typically, this technique involves immunizing a suitable animal with a selected immunogen composition, *e.g.*, purified or partially purified protein, synthetic

protein or fragments thereof, as discussed in the section on polypeptides. Animals to be immunized are mammals such as cats, dogs, and horses, although there is no limitation other than that the subject be capable of mounting an immune response of some kind. The immunizing composition is administered in a manner effective to stimulate antibody producing cells. Rodents such as mice and rats are preferred animals. However, the use of rabbits, sheep or frogs is possible. The use of rats may provide certain advantages, but mice are preferred, with the BALB/c mouse being most preferred as the most routinely used animal and one that generally gives a higher percentage of stable fusions.

For generation of monoclonal antibodies (MAbs), following immunization, somatic cells with the potential for producing antibodies, specifically B lymphocytes (B cells), are selected for use in the MAb generating protocol. These cells may be obtained from biopsied spleens, tonsils or lymph nodes, or from a peripheral blood sample. Spleen cells and peripheral blood cells are preferred, the former because they are a rich source of antibody-producing cells that are in the dividing plasmablast stage, and the latter because peripheral blood is easily accessible. Often, a panel of animals will have been immunized and the spleen of the animal with the highest antibody titer removed. Spleen lymphocytes are obtained by homogenizing the spleen with a syringe. Typically, a spleen from an immunized mouse contains approximately 5×10^7 to 2×10^8 lymphocytes.

The antibody-producing B cells from the immunized animal are then fused with cells of an immortal myeloma cell line, generally one of the same species as the animal that was immunized. Myeloma cell lines suited for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency and enzyme deficiencies that render them incapable of growing in certain selective media which support the growth of only the desired fused cells, called "hybridomas."

Any one of a number of myeloma cells may be used and these are known to those of skill in the art. For example, where the immunized animal is a mouse, one may use P3-X63/Ag8, X63-Ag8.653, NS1/1.Ag 41, Sp210-Ag14, FO, NSO/U, MPC-11,

MPC11-X45-GTG 1.7 and S194/5XX0 Bul; for rats, one may use R210.RCY3, Y3-Ag 1.2.3, IR983F and 4B210; and U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6 are all useful in connection with human cell fusions.

One preferred murine myeloma cell line is the NS-1 myeloma cell line (also termed P3-NS-1-Ag4-1), which is readily available from the NIGMS Human Genetic Mutant Cell Repository by requesting cell line repository number GM3573. Another mouse myeloma cell line that may be used is the 8-azaguanine-resistant mouse murine myeloma SP2/0 non-producer cell line.

Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually comprise mixing somatic cells with myeloma cells in a 2:1 proportion, though the proportion may vary from about 20:1 to about 1:1, respectively, in the presence of an agent or agents (chemical or electrical) that promote the fusion of cell membranes. Fusion methods using Sendai virus have been described by Kohler & Milstein (1975; 1976), and those using polyethylene glycol (PEG), such as 37% (v/v) PEG, by Gefter *et al.* (1977). The use of electrically induced fusion methods is also appropriate.

Fusion procedures usually produce viable hybrids at low frequencies, from about 1×10^{-6} to 1×10^{-8}. This does not pose a problem, however, as the viable, fused hybrids are differentiated from the parental, unfused cells (particularly the unfused myeloma cells that would normally continue to divide indefinitely) by culture in a selective medium. The selective medium generally is one that contains an agent that blocks the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are aminopterin, methotrexate. and azaserine. Aminopterin and methotrexate block *de novo* synthesis of both purines and pyrimidines, whereas azaserine blocks only purine synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides (HAT medium). Where azaserine is used, the media is supplemented with hypoxanthine.

The preferred selection medium is HAT. Only cells capable of operating nucleotide salvage pathways are able to survive in HAT medium. The myeloma cells are defective in key enzymes of the salvage pathway, *e.g.*, hypoxanthine phosphoribosyl transferase (HPRT), and they cannot survive. The B cells can operate this pathway, but they have a limited life span in culture and generally die within about two weeks. Therefore, the only cells that can survive in the selective media are those hybrids formed from myeloma and B cells.

This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants (after about two to three weeks) for the desired reactivity. The assay should be sensitive, simple, and rapid, such as radioimmunoassays, enzyme immunoassays, cytotoxicity assays, plaque assays, dot immunobinding assays, and the like.

The selected hybridomas are then serially diluted and cloned into individual antibody-producing cell lines, which clones can then be propagated indefinitely to provide MAbs. The cell lines may be exploited for MAb production in two basic ways. A sample of the hybridoma can be injected, usually in the peritoneal cavity, into a histocompatible animal of the type that was used to provide the somatic and myeloma cells for the original fusion. The injected animal develops tumors secreting the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can then be tapped to provide MAbs in high concentration. The individual cell lines could also be cultured *in vitro*, where the MAbs are naturally secreted into the culture medium from which they can be readily obtained in high concentrations. MAbs produced by either means may be further purified, if desired, using filtration, centrifugation, and various chromatographic methods such as HPLC or affinity chromatography.

Monoclonal antibodies of the present invention also include anti-idiotypic antibodies produced by methods well-known in the art. Monoclonal antibodies according

to the present invention also may be monoclonal heteroconjugates, *i.e.*, hybrids of two or more antibody molecules. In another embodiment, monoclonal antibodies according to the invention are chimeric monoclonal antibodies. In one approach, the chimeric monoclonal antibody is engineered by cloning recombinant DNA containing the promoter, leader, and variable-region sequences from a mouse antibody producing cell and the constant-region exons from a human antibody gene. The antibody encoded by such a recombinant gene is a mouse-human chimera. Its antibody specificity is determined by the variable region derived from mouse sequences. Its isotype, which is determined by the constant region, is derived from human DNA.

In another embodiment, monoclonal antibodies according to the present invention is a "humanized" monoclonal antibody, produced by techniques well-known in the art. That is, mouse complementary determining regions ("CDRs") are transferred from heavy and light V-chains of the mouse Ig into a human V-domain, followed by the replacement of some human residues in the framework regions of their murine counterparts. "Humanized" monoclonal antibodies in accordance with this invention are especially suitable for use in *in vivo* diagnostic and therapeutic methods.

As stated above, the monoclonal antibodies and fragments thereof according to this invention can be multiplied according to *in vitro* and *in vivo* methods well-known in the art. Multiplication *in vitro* is carried out in suitable culture media such as Dulbecco's modified Eagle medium or RPMI 1640 medium, optionally replenished by a mammalian serum such as fetal calf serum or trace elements and growth-sustaining supplements, *e.g.*, feeder cells, such as normal mouse peritoneal exudate cells, spleen cells, bone marrow macrophages or the like. *In vitro* production provides relatively pure antibody preparations and allows scale-up to give large amounts of the desired antibodies. Techniques for large scale hybridoma cultivation under tissue culture conditions are known in the art and include homogenous suspension culture, *e.g.*, in an airlift reactor or in a continuous stirrer reactor or immobilized or entrapped cell culture.

Large amounts of the monoclonal antibody of the present invention also may be obtained by multiplying hybridoma cells *in vivo*. Cell clones are injected into mammals which are histocompatible with the parent cells, *e.g.*, syngeneic mice, to cause growth of antibody-producing tumors. Optionally, the animals are primed with a hydrocarbon, especially oils such as Pristane (tetramethylpentadecane) prior to injection.

In accordance with the present invention, fragments of the monoclonal antibody of the invention can be obtained from monoclonal antibodies produced as described above, by methods which include digestion with enzymes such as pepsin or papain, and/or cleavage of disulfide bonds by chemical reduction. Alternatively, monoclonal antibody fragments encompassed by the present invention can be synthesized using an automated peptide synthesizer, or they may be produced manually using techniques well known in the art.

The monoclonal conjugates of the present invention are prepared by methods known in the art, *e.g.*, by reacting a monoclonal antibody prepared as described above with, for instance, an enzyme in the presence of a coupling agent such as glutaraldehyde or periodate. Conjugates with fluorescein markers are prepared in the presence of these coupling agents, or by reaction with an isothiocyanate. Conjugates with metal chelates are similarly produced. Other moieties to which antibodies may be conjugated include radionuclides such as ^{3}H, ^{125}I, ^{131}I ^{32}P, ^{35}S, ^{14}C, ^{51}Cr, ^{36}Cl, ^{57}Co, ^{58}Co, ^{59}Fe, ^{75}Se, ^{152}Eu, and ^{99m}Tc, are other useful labels which can be conjugated to antibodies. Radioactively labeled monoclonal antibodies of the present invention are produced according to well-known methods in the art. For instance, monoclonal antibodies can be iodinated by contact with sodium or potassium iodide and a chemical oxidizing agent such as sodium hypochlorite, or an enzymatic oxidizing agent, such as lactoperoxidase. Monoclonal antibodies according to the invention may be labeled with technetium-^{99m} by ligand exchange process, for example, by reducing pertechnate with stannous solution, chelating the reduced technetium onto a Sephadex column and applying the antibody to this column or by direct labeling techniques, *e.g.*, by incubating pertechnate, a reducing agent

such as $SNCl_2$, a buffer solution such as sodium-potassium phthalate solution, and the antibody.

F. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

Determination of the Crystal Structure of eNOS

Crystals were grown as described. Briefly, bovine eNOS heme domain (39-482; Mr 49,000) was obtained *via* trypsinolysis of a modified version of the holo eNOS construct (expressed in *E. coli)* missing 75% of the calmodulin binding region. Crystals suitable for diffraction were grown by the sitting drop vapor-diffusion method from 15% PEG 3350, 200 mM magnesium acetate, 100 mM sodium cacodylate, pH 6.5, and 2 mM *S*-ethylisothiourea (or 10 mM L-Arg) in the presence of 75 μM sodium dodecyl sulfate (SDS) as an additive and 10 mM tris(2-carboxyethyl)phosphine (TCEP) or 5 mM glutathione sulfonate as reducing agent. No BH_4 was added during crystallization. Pterin-free protein expressed in *E. coli* was purified either in the presence (50 μM) or absence of BH_4 towards obtaining crystals with cofactor-bound and -free forms. Crystals grown under these conditions belong to the orthorhombic space group, $P2_12_12_1$, with cell constants a=58.00 Å, b=106.55 Å, and c=156.22 Å. There is one dimer in the asymmetric unit (50% solvent content). All native and derivative crystals were flash frozen in liquid nitrogen for both storage and data collection at cryogenic temperatures (100 K). A

protein stabilization cocktail containing 15% glycerol, 11% trehalose, 8% mannitol and 8% sucrose was used as cryoprotectant.

Data were collected with a charge coupled device (CCD) detector at CHESS, NSLS and SSRL (beamlines Fl and F2, X12B and 1-5, respectively) and with a Mar Research image plate scanner at SSRL (beamlines 7-1 and 9-1). Multiwavelength anomalous diffraction (MAD) data were collected using the inverse beam mode after aligning the crystal with a major axis coincident with the rotation axis so that Bijvoet pairs could be measured simultaneously. Image plate data were reduced using the programs DENZO and SCALEPACK (Otwinowski and Minor, 1997) and CCD data were processed with the DPS, MOSFLM and CCP4 suite of programs. Mercury and osmium positions (three sites each) were readily identified by SHELXS (Sheldrick, 1997) and could later be confirmed in the isomorphous and anomalous difference Patterson maps. Iterative rounds of rejections performed with ENDHKL (Louis Sanchez, Cal. Tech.) in conjunction with SCALEPACK and local scaling were both critical for the identification of the heavy atoms. For MAD phasing an inhibitor, S-(2-(5((amidinothio)methyl)-2-thienyl)ethyl)isothiourea (Garvey *et al.,* 1994), was prepared with the sulfur atoms replaced with selenium. After 15 min, a solution of 2.401 g (0.011 mol) of ethyl 2-selenophenacetate in 5 ml of dichlormethane was added dropwise over several minutes. The mixture was poured into water and ice after 1 h and stirred for 30 min. The dichlormethane layer was washed with water, dried over sodium sulfate, and concentrated. The crude product was purified by silica gel chromatography with 10% ethyl acetate in petrolether to yield 1.77 g (65%) of 5-formyl-2-selenophenacetic acid ethyl esther intermediate. H NMR (300 MHz, CDC13) 9.83 (formyl, 1H), 7.63-7.04 (aromatic, 2H), 4.19-4.17 (methyl, 5H), 3.86 (ethyl, 1H), 1.28-1.24 (methyl, 5H). To a 0 oC stirred suspension of 0.77 g (20.29 mmol) of lithium aluminium hydride (Aldrich) in 200 ml of tetrahydrofuran was added a solution of 2.0 g (8.65 mmol) of the intermediate prepared above in tetrahydrofuran. The suspension was stirred at 20 oC for 16 h, cooled to 0 oC, and the exess hydride was quenched by the carefull addition of 0.8 ml of water, 0.8 ml of 1N sodium hydroxyde solution, and 2.4 ml of water. The suspension was stirred with magnesium sulfate, filtered, concentrated, and purified by silica gel

chromatigraphy with 50% ethyl acetate in petrolether. There was isolated 1.0 g (66%) of diol intermediate (yellow oil). H NMR (300 MHz, CDC13) 797- 7.13 (aromatic, 2H), 4.83 (hydroxyl, 2H), 3.63 (ethyl, 5H), 1.63-1.42 (ethyl, 1H). A solution of this diol (1 g, 5.6 mmol) in dichlormethane (20 ml) at 0 oC was treated with 3.1 g (9.7 mmol) of carbon tribromide and 2.5 g (9.7 mmol) triphenylphosphine. The mixture was stirred at 20 oC for 4 h before 50 ml of petrolether was added. After 15 h, the solution was decanted from brown-colored solid, concentrated, and purified by silica gel chromatography (ethyl acetate/petrolether, 80:20) to yield 0.7 g (63%) of dibromide as an oil. A solution of 0.7 g (2.3 mmol) of dibromide and 1 g (8.12 mmol) of selenourea in 20 ml of absolute ethanol was refluxed for 2 h, cooled and concentrated to dryness. The crude solid was recristallized from ethanol to yield 0.1 g (9.4%) of the bis-ISU as a yellow crystalline solid. H NMR (300 MHz, CDCL3) 9.25 (NH, 6H), 6.2 (aromatic, 2H), 4.22-4.15 (ethyl, 4H), 1.23-1.13 (ethyl, 4H).

The preparation of 2-selenophenacetate:

To a solution of 3.18 g ethylbromoacetate and 0.362 g of tin (IV) chloride in 10 ml of carbon disulfide at 0 oC was added dropwise over several minutes 2.5 g of selenophene in 1 ml CS2. The mixture was stirred at room temperature ovemight, poured into water and ice, extracted by dichlommethane, washed by saturated solution of sodium hydrocarbonate, dried over sodium sulfate and concentrated. The crude product was purified by silica gel chromatography in dichlormethane to yield 0.7 g ethyl 2-sdenophenacetate (18.3%). 1H NMR (300 MHz, DMSO) 7.23-7.2 (1H), 6.97-6.94(2H), 4.22-4.15 (ethyl 5H), 3.83 (methylen, 2H), 1.29-1.25 (methyl, 5H).

Once the structure was refined, it was evident that the inhibitor had not bound. One of the six sites initially assigned to selenium was the new zinc site and two others are most likely adducts of $(CH_3)_2As$ to both Cys384 residues (one per monomer) from the cacodylate buffer used in crystallization. Since the crystallization solution contains excess reducing agents, the inventors attribute this chemistry to the reduction of dimethylarsenate (V) to dimethylarsenite (III) followed by reaction with Cys384 (Barber, 1932; Tsao and Maki, 1991). The three remaining sites initially thought to be selenium,

two near both Cys214 sulfurs and one near one Cys87, were much weaker and could not be confidently modeled as $(CH_3)_2As$ sites in the final refined electron density map. Despite the incorrect assignment of the arsenic and zinc sites as selenium, their inclusion was essential for obtaining an interpretable electron density map. To the best of the inventors' knowledge this is the first study where arsenic atoms have been used successfully in phasing. Availability of heme iron positions, identified independently *via* anomalous scattering at the Fe edge, greatly facilitated the location of heavy atom sites. Heavy atom derivative screening and preliminary phase refinements were carried out with PHASES and visualized using XTALVIEW.

The final combined MAD and heavy atom refinement was done with SHARP (de La Fortelle and Bricogne, 1997) followed by density modification with either SOLOMON (Abrahams and Leslie, 1996) or DM. The latter calculation includes non-crystallographic symmetry (TICS) averaging. The resulting experimental MAD-heavy atom map at 2.35 Å was of sufficient quality to allow nearly all main chain atoms and 80% of the side chain atoms to be built into the model before the first round of refinement. Phase refinement with heavy atom derivatives alone did not produce an interpretable map. Heme iron positions, SHELXS, MAD phasing, and SHARP were a *sine qua non* for success in the structure solution.

Structural refinement was performed with XPLOR (Brünger, 1992) and SHELXL (Sheldrick and Schneider, 1997). Five percent of the data were set aside for free-R cross validation prior to any structural refinement. The protein model was built using TOM and improved with SigmaA-weighted $2|Fobs| - |Fcalc|$ and $|Fobs| - |Fcalc|$ maps iteratively with X-PLOR refinement. A bulk solvent correction was used in the final stages of the X-PLOR but not in the SHELXL refinement. The current model at 1.9 Å resolution includes 830 residues (residues 67 - 482 in molecule A; 69 - 482 in molecule B) and 591 waters. Residues 39-66 and 108-121 are disordered primarily due to the proline-rich nature of this region. Ramachandran plots generated with PROCHECK showed that 88.9% of the residues were in the most favored regions, 11% in additional

allowed regions and 0. 1% in disallowed regions. Solvent accessible surface area calculations were done with MSP.

Table 5

Data collection	Native	H4 free	Se edge	Se-peak	Se- remote	Se-EMP*	Se- $OsO_3(PY)_2$*
Wavelength (Å)	0.9798	1.08	0.9801	0.9794	0.9252	1.08	1.08
Resolution limits (Å)	1.9	2.1	2.3	2.3	2.3	2.3	3.0
Total observations	217,080	204,608	131,275	127,968	144,570	131,287	74,034
Unique observations	74,337	57,723	40,100	36,351	40,965	40,508	16,204
R_{sym}	0.044	0.084	0.048	0.054	0.044	0.059	0.085
R_{sym} (outer shell)	0.175	0.563	0.121	0.219	0.185	0.325	0.336
$<I/\sigma>$	19.3	7.7	16.9	15.3	17.1	9.8	8.9
$<I/\sigma>$ (outer shell)	3.5	2.4	3.2	2.2	3.1	2.2	4.0
Completeness	0.965	0.996	0.962	0.988	0.985	0.977	0.808
Completeness (outer shell)	0.800	0.996	0.795	0.802	0.983	0.801	0.829
MAD phasing (20.0 - 2.35 Å)							
Number of sites			6				
Phasing power Iso/Ano			0.0/0.76	0.0/0.78	1.1/0.75		
R_{Cullis} Iso/Ano			0.0/0.96	0.97/0.96	0.41/0.95		
MIRAS phasing							
Number of sites						6	5
R_{iso}						0.157	0.173
R_{cullis}						0.89	0.92
Phasing power Iso/Ano						1.15/0.87	0.71/0.85
Overall FOM (MAD+MIRAS)			0.29 (2.35 Å)				
Refinement	Resolution (Å)	Protein	Waters	R-factor	R-free	Reflections	R.m.s. deviation[§]

Data collection	Native	H4 free atoms	Se edge	Se-peak	Se- remote	Se-EMP*	Se- $OsO_3(PY)_2$*
X-PLOR (native)	30.0 - 1.9	6593	591	0.225 (F>2σF)	0.262	73,483 (F>2σF)	
SHELXL (native)	10.0 - 1.9	6593	591	0.207	0.278	70,029	0.007Å 0.021Å
X-PLOR (BH_4 free)	30.0 - 2.1	6593	336	0.187 (F>2σF)	0.247	48,347 (F>2σF)	0.007Å 1.408°

$R_{sym} = \Sigma|I - \langle I\rangle/\Sigma$, where I is the observed intensity and $\langle I\rangle$ the average intensity of multiple symmetry-related observations of that reflection. $R_{iso} = \Sigma|F_{PH} - F_p|/\Sigma F_p$. R_{cullis} ISO (acentric) = r.m.s. lack of closure / r.m.s. isomorphous difference (statistics from SHARP). R_{cullis} Ano = r.m.s. lack of closure / r.m.s. anomalous difference (statistics from SHARP). Phasing power = r.m.s. heavy atom structure factor / phase integrated lack of closure (statistics from SHARP). Overall FOM = overall figure of merit (from SHARP). R-free = R-factor calculated using 5% (3685 reflections) of the reflection data chosen randomly and set aside from the start of the refinement. EMP = ethyl mercuric phosphate. OsO_3Py_2 = osmium bis-pyridine. [§] r.m.s. deviations are defined as bond length and angular distances in SHELXL (Sheldrick and Schneider, 1997) and bond length and bond angle, respectively, in X-PLOR (Brünger, 1992).

The inventors report the 1.9 Å crystal structure of the dimeric heme domain of constitutive eNOS (Table 5), which maintains the catalytic site for $NO^{\bullet}$ synthesis. The overall fold of the bovine eNOS heme domain (FIG. 1) is similar to that reported for the 2.6 Å structure of the iNOS heme domain dimer (Crane *et al.*, 1998). The quaternary structure of eNOS is characterized by a tightly packed dimer interface which buries 3000 $Å^2$ per subunit of solvent accessible surface (55% hydrophobic and 45% polar).

The inventors solved the structure of eNOS heme domain both in the presence and absence of BH_4 resulting in three major structural findings. First, the inventors found a novel ZnS_4 center (in both pterin-bound and -free structures) located at the bottom of the dimer interface (FIG. 1) with the metal tetrahedrally coordinated by two pairs of symmetry-related cysteine residues (FIG. 2) from each subunit.

Second, in the absence of BH_4, the overall eNOS heme domain structure remains unchanged both in the tertiary topology and quaternary structure. These results sharply contrast with the conclusions of Crane *et al.*, (1998) who proposed that large conformational changes concomitant with BH_4 binding were necessary for inducible NOS dimerization. By comparison of the pterin-bound and -free structures, it is clear that the site preexists (FIG. 3) and does **not** form *via* an induced fit process. The pterin-free structure also does not show any major changes at the active site either in the substrate- or inhibitor (*S*-ethylisothiourea, SEITU)-bound conformations.

Third, in addition to the new ZnS_4 center, the most striking and novel finding is the identification of an L-Arg bound to the pterin site in the absence of BH_4 (FIG. 3A and FIG. 3D). The mode of L-Arg interaction at the pterin site surprisingly mimics that entertained by BH_4 itself. Two crucial H-bonds, one between a guanidino N with the heme propionate and that between the primary amino group and Ser 104 are strictly conserved (FIG. 3D). Moreover, the planar guanidinium group is sandwiched between two aromatic groups, one from each monomer, exactly as in the BH_4 complex. Solvent interactions also are similar to the BH_4 complex. The affinity L-Arg must be great since

no exogenous L-Arg was added during crystallization. The specificity of L-Arg recognition at the pterin site appears to require both the guanidino function and the primary amino group since SEITU was unable to displace L-Arg from the pterin site. The relevance of these findings is discussed in greater detail herein above.

* * *

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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WHAT IS CLAIMED IS:

1. A method for the structural analysis of endothelial nitric oxide synthase comprising:

   a) subcloning a gene encoding the endothelial nitric oxide synthase in an expression vector;
   b) obtaining expression of the endothelial nitric oxide synthase protein from the vector;
   c) purifying the endothelial nitric oxide synthase protein;
   d) preparing an endothelial nitric oxide synthase protein sample amenable for crystallization;
   e) crystallizing the endothelial nitric oxide synthase protein sample; and
   f) performing a three-dimensional structural analysis of the endothelial nitric oxide synthase by x-ray crystallography.

2. The method of claim 1, wherein the endothelial nitric oxide synthase protein expressed is a variant endothelial nitric oxide synthase protein.

3. The method of claim 1, wherein the crystallizing comprises practicing a sitting drop vapor-diffusion method.

4. The method of claim 1, wherein the crystallizing is performed in the presence of tetrahydrobiopterin.

5. The method of claim 1, wherein the crystallizing is performed in the absence of tetrahydrobiopterin.

6. The method of claim 1, wherein the x-ray crystallography is performed in the presence of tetrahydrobiopterin.

7. The method of claim 1, wherein the x-ray crystallography is performed in the absence of tetrahydrobiopterin.

8. A method of structural analysis to determine the binding of pterin to endothelial nitric oxide synthase comprising:

a) determining the crystal structure of a dimeric heme domain of the endothelial nitric oxide synthase in the presence of the pterin; and

b) determining the crystal structure of a dimeric heme domain of the endothelial nitric oxide synthase in the absence of the pterin;

and comparing the crystal structures.

9. The method of claim 8, wherein the pterin is tetrahydrobiopterin.

10. The method of claim 9, wherein the tetrahydobiopterin is (1'R,2'S,6R)-5,6,7,8-tetrahydrobiopterin.

11. The method of claim 8, wherein the crystal structure is determined at a resolution of between about 1.9 Å to about 2.1 Å.

12. A method for screening and identifying small molecule modulators of endothelial nitric oxide synthase proteins comprising:

a) providing a pterin-free endothelial nitric oxide synthase structure;

b) screening the small molecule modulators for their ability to bind to a pterin-binding site of the endothelial nitric oxide synthase ; and

c) performing assays to determine the ability of the small molecule modulators to modulate the activity of endothelial nitric oxide synthase.

13. The method of claim 12, wherein the small molecule modulator inhibits endothelial nitric oxide synthase.

14. The method of claim 12, wherein the small molecule modulator activates endothelial nitric oxide synthase.

15. The method of claim 12, wherein the endothelial nitric oxide synthase protein expressed is a variant endothelial nitric oxide synthase protein.

16. The method of claim 12, wherein the pterin is tetrahydrobiopterin.

17. The method of claim 16, wherein the tetrahydobiopterin is (1'R,2'S,6R)-5,6,7,8-tetrahydrobiopterin.

18. The method of claim 12, wherein the small molecule modulators are molecules and chemical-fragments from chemical-fragment libraries.

19. The method of claim 12, wherein the screening is performed by computerized methods.

20. The method of claim 12, wherein the assays are performed *in vitro* or *in vivo*.

21. A method for identifying drugs against diseased states in which nitric oxide signaling is defective or insufficient comprising:

a) providing a tetrahydrobiopterin-free endothelial nitric oxide synthase structure;

b) screening the drugs for their ability to bind the tetrahydrobiopterin binding site; and

c) performing assays to determine the ability of the drugs to activate the endothelial nitric oxide synthase.

22. The method of claim 21, wherein the diseased states include impaired neurotransmission; impaired insulin release; impaired penile erection; impaired vasorelaxation; and impaired oxygen detection.

23. Endothelial nitric oxide synthase structure, obtained by the process comprising:

   a) subcloning a gene encoding the endothelial nitric oxide synthase in an expression vector;
   b) obtaining expression of the endothelial nitric oxide synthase protein from the vector;
   c) purifying the endothelial nitric oxide synthase protein;
   d) preparing an endothelial nitric oxide synthase protein sample amenable for crystallization;
   e) crystallizing the endothelial nitric oxide synthase protein sample.
   f) performing a three-dimensional structural analysis of the endothelial nitric oxide synthase by x-ray crystallography.

24. A nitric oxide synthase obtained according to the method of claim 1.

25. A method for screening and identifying a candidate substance with the ability to inhibit endothelial nitric oxide synthase comprising:

   a) obtaining a cell with endothelial nitric oxide synthase activity;
   b) admixing the candidate substance with the cell; and
   c) determining the ability of the candidate substance to inhibit the endothelial nitric oxide synthase activity of the cell.

26. The method of claim 25, wherein the candidate substance is a small molecule modulator of endothelial nitric oxide synthase.

27. A method for screening and identifying a candidate substance with the ability to inhibit endothelial nitric oxide synthase comprising:

   a) obtaining a purified endothelial nitric oxide synthase;
   b) admixing the endothelial nitric oxide synthase with the candidate substance; and

c) performing X-ray crystallography analysis to determine the binding of the candidate substance.

Figure 1

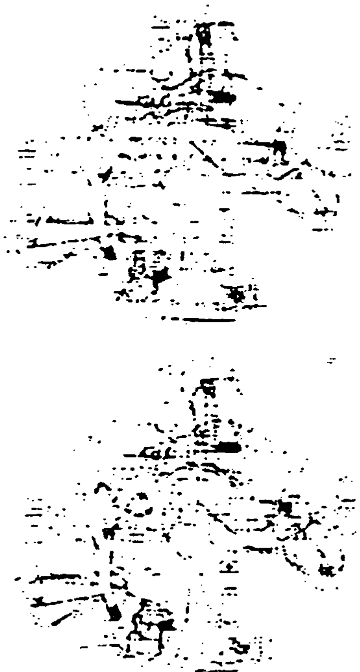


Figure 2

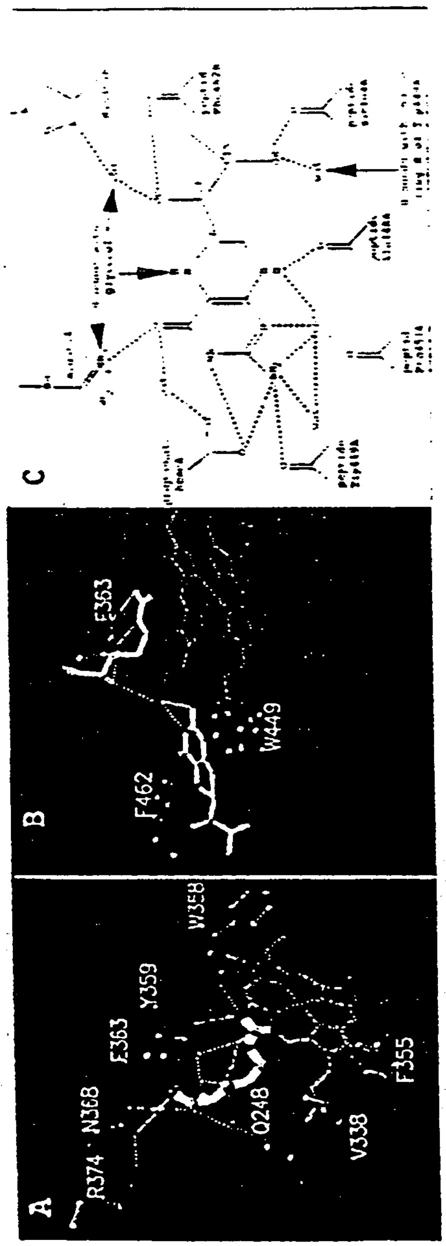


Figure 3

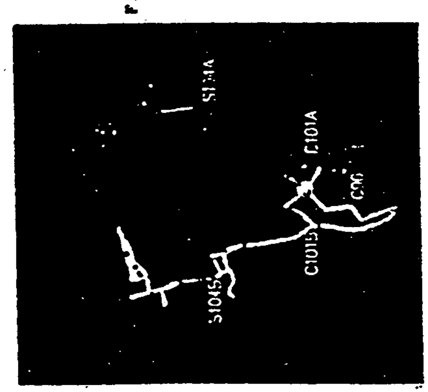
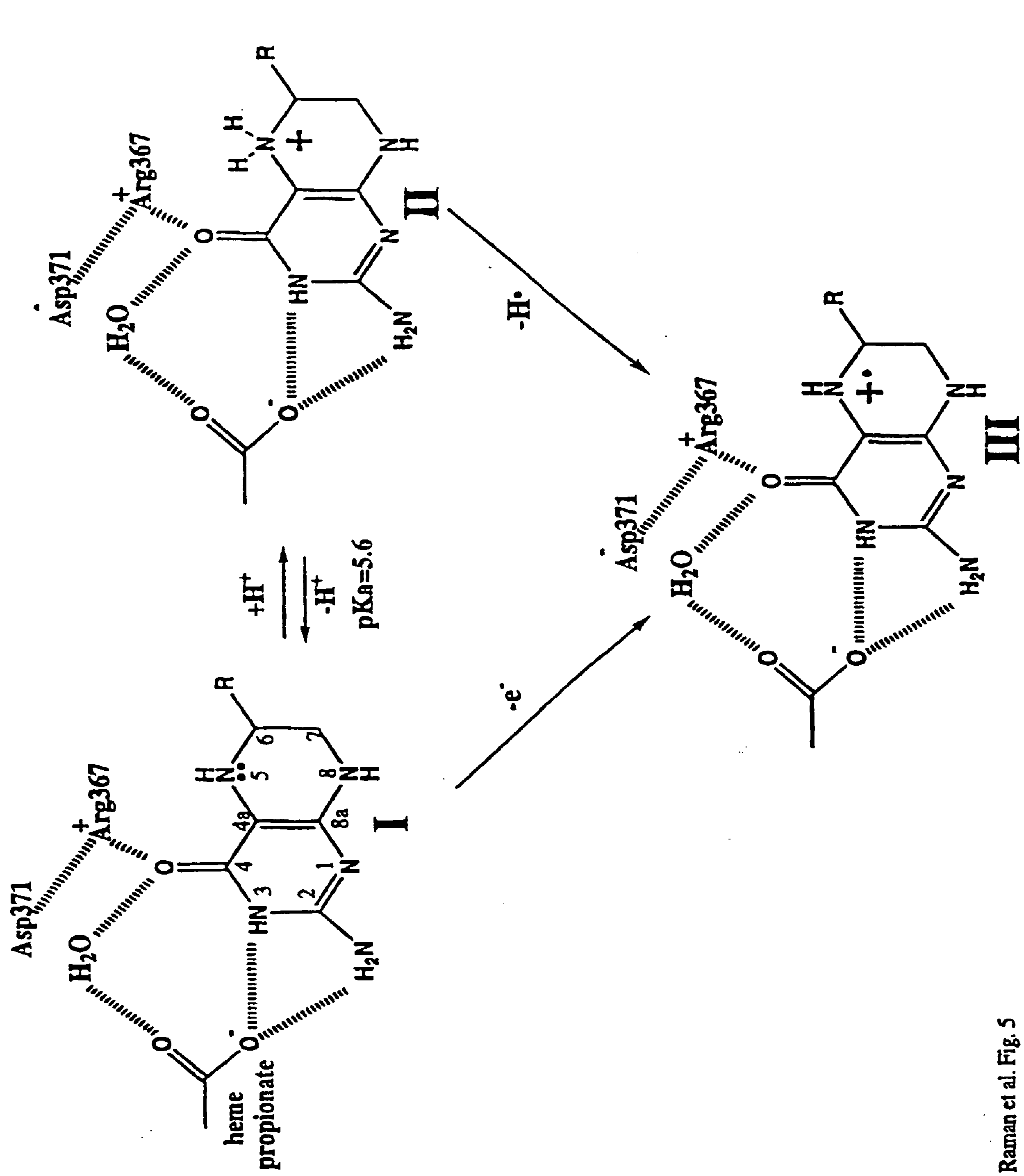


Figure 4

Figure 5



Raman et al. Fig. 5

INTERNATIONAL SEARCH REPORT

Int. ..tional Application No
PCT/US 99/30707

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/53 C12N9/02 C12Q1/26

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X	WO 93 18156 A (GEN HOSPITAL CORP) 16 September 1993 (1993-09-16)	24
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 May 2000	23/05/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hornig, H

2

International Application No
PCT/US 99/30707

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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X Y	US 5 498 539 A (HARRISON DAVID G ET AL) 12 March 1996 (1996-03-12) the whole document	24 1-11
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Inte .ional Application No
PCT/US 99/30707

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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P,X	FISCHMANN THIERRY O ET AL: "Structural characterization of nitric oxide synthase isoforms reveals striking active-site conservation." NATURE STRUCTURAL BIOLOGY MARCH, 1999, vol. 6, no. 3, March 1999 (1999-03), pages 233-242, XP002137149 ISSN: 1072-8368 the whole document -----	1-11,23, 24

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 99/30707

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9318156 A	16-09-1993	AU 3789193 A	05-10-1993
US 5498539 A	12-03-1996	NONE	
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		AU 6910296 A	19-03-1997
WO 9802555 A	22-01-1998	AU 3885497 A	09-02-1998
		EP 0938567 A	01-09-1999
WO 9641885 A	27-12-1996	US 5744340 A	28-04-1998
		AU 6149296 A	09-01-1997
		CA 2224089 A	27-12-1996
		EP 0832246 A	01-04-1998
		JP 11507830 T	13-07-1999

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/30707

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/53 C12N9/02 C12Q1/26

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/30707

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INTERNATIONAL SEARCH REPORT

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PCT/US 99/30707

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
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			EP	0832246	A	01-04-1998
			JP	11507830	T	13-07-1999

PATENT COOPERATION TREATY

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INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference UTFK336P	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/US 99/30707	International filing date *(day/month/year)* 22/12/1999	(Earliest) Priority Date *(day/month/year)* 22/12/1998
Applicant BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

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b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

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[ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

[X] the text is approved as submitted by the applicant.

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5. With regard to the **abstract**,

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 2

[ ] as suggested by the applicant.

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[ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/30707

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International Application No
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INTERNATIONAL SEARCH REPORT

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			CA	2224089	A	27-12-1996
			EP	0832246	A	01-04-1998
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PCT REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty

For receiving Office use only
International Application No.
International Filing Date
Name of receiving Office and "PCT International Application"
Applicant's or agent's file reference *(if desired) (12 characters maximum)* UTFK336P

Box No. I TITLE OF INVENTION
STRUCTURAL BASIS FOR PTERIN FUNCTION IN NITRIC OXIDE SYNTHASE

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

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201 W. 7th St.
Austin, TX 78701
US

☐ This person is also inventor.

Telephone No.
Facsimile No.
Teleprinter No.

State *(that is, country)* of nationality: US | State *(that is, country)* of residence: US

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

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This person is:
☐ applicant only
☒ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: US | State *(that is, country)* of residence: US

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by give name; for a legal entity, full official designation. The address must include postal code and name of country.)*

WILSON, MARK B.
ARNOLD WHITE & DURKEE
750 Bering Drive
Houston, TX 77057-2198
United States of America

Telephone No. (713) 787-1400
Facsimile No. 713-787-1440
Teleprinter No.

☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Continuation of Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTORS

If none of the following sub-boxes is used, this sheet is not to be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

MARTASEK, Pavel
7531 Pipers Lane
San Antonio, TX 78251
US

This person is:
☐ applicant only
☒ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: CZ

State *(that is, country)* of residence: US

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

ROMAN, Linda J.
2814 Squaw Creek
San Antonio, TX 78230
US

This person is:
☐ applicant only
☒ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: US

State *(that is, country)* of residence: US

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

KRAL, Vladimir
Na Kozacce 8/9275
120 00 Prahal
Czech Republic

This person is:
☐ applicant only
☒ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: CZ

State *(that is, country)* of residence: CZ

This person is applicant for the purposes of: ☐ all designated States ☐ all designated Sates except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

POULOS, Thomas L.
48 Urey Court
Irvine, CA 92612
US

This person is:
☐ applicant only
☒ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence: US

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Continuation of Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTORS

If none of the following sub-boxes is used, this sheet is not to be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)* RAMAN, C.S.	This person is: ☐ applicant only ☒ applicant and inventor ☐ inventor only *(If this check-box is marked, do not fill in below.)*
State *(that is, country)* of nationality:	State *(that is, country)* of residence:
This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box	
Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*	This person is: ☐ applicant only ☐ applicant and inventor ☐ inventor only *(If this check-box is marked, do not fill in below.)*
State *(that is, country)* of nationality:	State *(that is, country)* of residence:
This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box	
Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*	This person is: ☐ applicant only ☐ applicant and inventor ☐ inventor only *(If this check-box is marked, do not fill in below.)*
State *(that is, country)* of nationality:	State *(that is, country)* of residence:
This person is applicant for the purposes of: ☐ all designated States ☐ all designated Sates except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box	
Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*	This person is: ☐ applicant only ☐ applicant and inventor ☐ inventor only *(If this check-box is marked, do not fill in below.)*
State *(that is, country)* of nationality:	State *(that is, country)* of residence:
This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box	
☐ Further applicants and/or (further) inventors are indicated on a continuation sheet.	

Box No. V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

☒ **AP ARIPO Patent: GH** Ghana, **GM** Gambia, **KE** Kenya, **LS** Lesotho, **MW** Malawi, **SD** Sudan, **SL** Sierre Leone, **SZ** Swaziland, **UG** Uganda, **ZW** Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT

☒ **EA Eurasian Patent: AM** Armenia, **AZ** Azerbaijan, **BY** Belarus, **KG** Kyrgyzstan, **KZ** Kazakstan, **MD** Republic of Moldova, **RU** Russian Federation, **TJ** Tajikistan, **TM** Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT

☒ **EP European Patent: AT** Austria, **BE** Belgium, **CH** and **LI** Switzerland and Liechtenstein, **CY** Cyprus, **DE** Germany, **DK** Denmark, **ES** Spain, **FI** Finland, **FR** France, **GB** United Kingdom, **GR** Greece, **IE** Ireland, **IT** Italy, **LU** Luxembourg, **MC** Monaco, **NL** Netherlands, **PT** Portugal, **SE** Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT

☒ **OA OAPI Patent: BF** Burkina Faso, **BJ** Benin, **CF** Central African Republic, **CG** Congo, **CI** Côte d'Ivoire, **CM** Cameroon, **GA** Gabon, **GN** Guinea, **GW** Guinea-Bissau, **ML** Mali, **MR** Mauritania, **NE** Niger, **SN** Senegal, **TD** Chad, **TG** Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* ....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

☒**AE** United Arab Emirates
☒**AL** Albania
☒**AM** Armenia
☒**AT** Austria
☒**AU** Australia
☒**AZ** Azerbaijan
☒**BA** Bosnia and Herzegovina
☒**BB** Barbados
☒**BG** Bulgaria
☒**BR** Brazil
☒**BY** Belarus
☒**CA** Canada
☒**CH and LI** Switzerland and Liechtenstein
☒**CN** China
☒**CR** Costa Rica
☒**CU** Cuba
☒**CZ** Czech Republic
☒**DE** Germany
☒**DK** Denmark
☒**DM** Dominica
☒**EE** Estonia
☒**ES** Spain
☒**FI** Finland
☒**GB** United Kingdom
☒**GE** Georgia
☒**GD** Grenada
☒**GH** Ghana
☒**GM** Gambia
☒**HR** Croatia
☒**HU** Hungary
☒**ID** Indonesia
☒**IL** Israel
☒**IN** India
☒**IS** Iceland
☒**JP** Japan
☒**KE** Kenya
☒**KG** Kyrgyzstan
☒**KP** Democratic People's Republic of Korea
☒**KR** Republic of Korea
☒**KZ** Kazakstan
☒**LC** Saint Lucia
☒**LK** Sri Lanka
☒**LR** Liberia
☒**LS** Lesotho
☒**LT** Lithuania
☒**LU** Luxembourg
☒**LV** Latvia
☒**MA** Morocco
☒**MD** Republic of Moldova
☒**MG** Madagascar
☒**MK** The former Yugoslav Republic of Macedonia
☒**MN** Mongolia
☒**MW** Malawi
☒**MX** Mexico
☒**NO** Norway
☒**NZ** New Zealand
☒**PL** Poland
☒**PT** Portugal
☒**RO** Romania
☒**RU** Russian Federation
☒**SD** Sudan
☒**SE** Sweden
☒**SG** Singapore
☒**SI** Slovenia.
☒**SK** Slovakia
☒**SL** Sierra Leone
☒**TJ** Tajikistan
☒**TM** Turkmenistan
☒**TR** Turkey
☒**TT** Trinidad and Tobago
☒**TZ** The United Republic of Tanzania
☒**UA** Ukraine
☒**UG** Uganda
☒**US** United States of America (CONT.)
☒**UZ** Uzbekistan
☒**VN** Viet Nam
☒**YU** Yugoslavia
☒**ZA** South Africa
☒**ZW** Zimbabwe

Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after issuance of this sheet:
☐ ....

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except the designations(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

Supplemental Box ***If the Supplemental Box is not used, this sheet should not be included in the request.***

*1. If, in any of the Boxes, the space is **insufficient** to furnish all the information: in such case, write "Continuation of Box No. ..." [indicate the number of the Box] and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient, in particular:*

*(i) **if more than two persons are involved as applicants and/or inventors and no "continuation sheet" is available:** in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below;*

*(ii) if, in Box No. II or in any of the sub-boxes of Box No. III, the indication **"the States indicated in the Supplemental Box" is checked:** in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;*

*(iii) if, in Box No. II or in any of the sub-boxes of Box No. III, **the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America:** in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor(s) and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;*

*(iv) if, in addition to the agent(s) indicated in Box No. IV, there are **further agents:** in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;*

*(v) if, in Box No. V, the name of any State (or OAPI) is accompanied by the indication **"patent of addition," or "certificate of addition,"** or if, in Box No. V, the name of the United States of America is accompanied by an indication **"continuation" or "continuation-in-part":** in such case, write "Continuation of Box V" and the name of each State involved (or OAPI), and after the name of each such State (or OAPI), the number of the parent title or parent application and the date of grant of the parent title or filing of the parent application;*

*(vi) if, in Box No. VI, there are **more than three earlier applications whose priority is claimed:** in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI;*

*(vii) if, in Box No. VI, **the earlier application is an ARIPO application:** in such case, write "Continuation of Box VI", specify the number of the item corresponding to that earlier application and indicate at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed.*

*2. If, with regard to the **precautionary designation statement** contained in Box No. V, the applicant wishes to exclude any State(s) from the scope of that statement: in such case, write "Designation(s) excluded from precautionary designation statement" and indicate the name or two-letter code of each State so excluded.*

*3. If the applicant claims, in respect of any designated Office, the benefits, of provisions of the national law concerning **non-prejudicial disclosures or exceptions to lack of novelty:** in such case, write "Statement concerning non-prejudicial disclosures or exceptions to lack of novelty: and furnish that statement below.*

CONTINUATION OF BOX V, U.S. Serial No. 60/113.204 filed 22 December 1998 (22.12.98)

Box No. VI PRIORITY CLAIM ☐ Further priority claims are indicated in the Supplemental Box

Filing Date of earlier application *(day/month/year)*	Number of earlier application	Where earlier application is: national application: country	regional application:* regional Office	international application: receiving Office
item (1) 22 DECEMBER 1998 (22.12.98)	60/113.204	US		
item (2)				
item (3)				

☒ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s): EP

* *Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.*

BOX No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(If two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):*

ISA EPO

Request to use results of earlier search; reference to that search (if an earlier search has been carried out by or requested from the International Searching Authority):
Date (day/month/year) Number Country (or regional Office):

BOX No. VIII CHECK LIST; LANGUAGE OF FILING

This international application contains the following **number of sheets**:

request	: 5	sheets
description (excluding sequence listing part)	: 47	sheets
claims	: 5	sheets
abstract	: 1	sheets
drawings	: 2	sheets
sequence listing part of description	:	sheets
Total number of sheets:	60	sheets

This international application is **accompanied by** the item(s) marked below:

1. ☒ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☐ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ priority document(s) *identified in Box No. VI as item(s):*
6. ☐ translation of international application into (language):
7. ☐ separate indications concerning deposited microorganisms or other biological material
8. ☐ nucleotide and/or amino acid sequence listing in computer readable form
9. ☒ other (specify): **post card**

Figure of the drawings which should accompany the abstract:

Language of filing of the international application: English

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

Mark B. Wilson, Applicant's Agent

12/22/99
Date

For receiving Office use only

1. Date of actual receipt of the purported international application:
2. Drawings:
[ ] received:
[ ] not received
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
4. Date of timely receipt of the required corrections under PCT Article 11(2):
5. International Searching Authority (if two or more are competent): ISA/
6. [ ] Transmittal of search copy delayed until search fee is paid

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

PCT/US99/30707

●TENT COOPERATION TRE●

PCT

NOTIFICATION OF RECEIPT OF RECORD COPY

(PCT Rule 24.2(a))

From the INTERNATIONAL BUREAU

To:

WILSON, Mark, B. REC'D HOWREY SIMON ARNOLD & WHITE
Arnold White & Durkee
750 Bering Drive
Houston, TX 77057-2198
ÉTATS-UNIS D'AMÉ HOUSTON DOCKETING DEPT.

MAR 16 2000

Date of mailing (day/month/year) 02 March 2000 (02.03.00)	**IMPORTANT NOTIFICATION**
Applicant's or agent's file reference UTFK336P	**International application No.** PCT/US99/30707

The applicant is hereby notified that the International Bureau has received the record copy of the international application as detailed below.

Name(s) of the applicant(s) and State(s) for which they are applicants:

BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM (for all designated States except US)
MASTERS, Bettie, Sue, S. et al (for US)

RECEIVED
F & J
AUSTIN INTL
MAR 20 2000

International filing date : 22 December 1999 (22.12.99)
Priority date(s) claimed : 22 December 1998 (22.12.98)
Date of receipt of the record copy by the International Bureau : 18 February 2000 (18.02.00)
List of designated Offices :

AP :GH,GM,KE,LS,MW,SD,SL,SZ,TZ,UG,ZW
EA :AM,AZ,BY,KG,KZ,MD,RU,TJ,TM
EP :AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE
OA :BF,BJ,CF,CG,CI,CM,GA,GN,GW,ML,MR,NE,SN,TD,TG
National :AE,AL,AM,AT,AU,AZ,BA,BB,BG,BR,BY,CA,CH,CN,CR,CU,CZ,DE,DK,DM,EE,ES,FI,GB,GD,GE,GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KP,KR,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MN,MW,MX,NO,NZ,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,US,UZ,VN,YU,ZA,ZW

FULBRIGHT & JAWORSKI LLP
AUSTIN, TEXAS
MAR 17 2000
RECEIVED

DOCKETED none
DATE
INITIALS ChC

DOCKETED ☐ UPDATED ☐
Previously ____ Not Required ✓
Appl. Info ____
Reg/Grant Info ____
Action Required: ____
Date Due/Done: ____
By: ____ Checked ____

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland** Facsimile No. (41-22) 740.14.35	Authorized officer: S. De Michiel Telephone No. (41-22) 338.83.38

Form PCT/IB/301 (July 1998)

003141563

DOCKETED ☐ UPDATED ☐
Previously X Not Required
Appl. Info
Reg/Client Info
Action Required:
Due Date/Done:
By: Checked

PCT/US99/30707

wim/stw

PATENT COOPERATION TREATY

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F & J
AUSTIN INTL
APR 17 2000

From the INTERNATIONAL BUREAU

PCT

To:

WILSON, Mark, B.
Arnold White & Durkee
750 Bering Drive
Houston, TX 77057-2198
ETATS-UNIS D'AMERIQUE

REC'D HOWREY SIMON ARNOLD & WHITE
APR 12 2000
HOUSTON DOCKETING DEPT.

NOTIFICATION CONCERNING SUBMISSION OR TRANSMITTAL OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

Date of mailing (day/month/year) 24 March 2000 (24.03.00)	
Applicant's or agent's file reference UTFK336P	**IMPORTANT NOTIFICATION**
International application No. PCT/US99/30707	International filing date (day/month/year) 22 December 1999 (22.12.99)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 22 December 1998 (22.12.98)
Applicant BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM et al	

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).

2. This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.

3. An **asterisk(*)** appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

4. The **letters "NR"** appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
22 Dece 1998 (22.12.98)	60/113,204	US	14 Marc 2000 (14.03.00)

FULBRIGHT & JAWORSKI LLP
AUSTIN, TEXAS
APR 14 2000
RECEIVED

DOCKETED none
DATE
INITIALS cre

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland** Facsimile No. (41-22) 740.14.35	Authorized officer Tessadel PAMPLIEGA Telephone No. (41-22) 338.83.38

Form PCT/IB/304 (July 1998)

003189217

PATENT COOPERATION TREATY

JUL 2 1 2000

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PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

From the INTERNATIONAL BUREAU

To:

WILSON, Mark, B.
Fulbright & Jaworski L.L.P.
600 Congress Avenue
Suite 2400
Austin, TX 78701
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year) 14 June 2000 (14.06.00)	
Applicant's or agent's file reference UTFK336P	**IMPORTANT NOTIFICATION**
International application No. PCT/US99/30707	**International filing date** (day/month/year) 22 December 1999 (22.12.99)

1. The following indications appeared on record concerning:

☐ the applicant ☐ the inventor ☒ the agent ☐ the common representative

Name and Address	State of Nationality	State of Residence
WILSON, Mark, B. Arnold White & Durkee 750 Bering Drive Houston, TX 77057-2198 United States of America		
	Telephone No. 713 787 1400	
	Facsimile No. 713 787 1440	
	Teleprinter No.	

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

☐ the person ☐ the name ☒ the address ☐ the nationality ☐ the residence

Name and Address	State of Nationality	State of Residence
WILSON, Mark, B. Fulbright & Jaworski L.L.P. 600 Congress Avenue Suite 2400 Austin, TX 78701 United States of America		
	Telephone No. 512 418 3000	
	Facsimile No. 512 474 7577	
	Teleprinter No.	

RECEIVED
F & J
AUSTIN INTL
JUL 24 2000

3. Further observations, if necessary:

DOCKETED
DATE noted
INITIALS CLC

4. A copy of this notification has been sent to:

☒ the receiving Office
☐ the International Searching Authority
☐ the International Preliminary Examining Authority
☐ the designated Offices concerned
☐ the elected Offices concerned
☐ other:

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland** Facsimile No.: (41-22) 740.14.35	Authorized officer S. De Michiel Telephone No.: (41-22) 338.83.38

003346838

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

To:

WILSON, Mark, B.
Fulbright & Jaworski L.L.P.
600 Congress Avenue
Suite 2400
Austin, TX 78701
ETATS-UNIS D'AMERIQUE

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

Date of mailing (day/month/year) 31 January 2001 (31.01.01)	
Applicant's or agent's file reference UTFK336P	**IMPORTANT NOTIFICATION**
International application No. PCT/US99/30707	**International filing date** (day/month/year) 22 December 1999 (22.12.99)

1. The following indications appeared on record concerning:

[X] the applicant [X] the inventor [ ] the agent [ ] the common representative

Name and Address	State of Nationality	State of Residence
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[X] the person [ ] the name [ ] the address [ ] the nationality [ ] the residence

Name and Address	State of Nationality	State of Residence
LI, Huiying 384 Falling Star Irvine, CA 92614 United States of America	CN	US
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

3. Further observations, if necessary:
Additional applicant and inventor for US only.

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland** Facsimile No.: (41-22) 740.14.35	Authorized officer C. Cupello Telephone No.: (41-22) 338.83.38

PCT/US99/30707

PATENT COOPERATION TREATY

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

From the INTERNATIONAL BUREAU

To:

WILSON, Mark, B.
Fulbright & Jaworski L.L.P.
600 Congress Avenue
Suite 2400
Austin, TX 78701
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year) 14 June 2000 (14.06.00)	
Applicant's or agent's file reference UTFK336P	**IMPORTANT NOTIFICATION**
International application No. PCT/US99/30707	**International filing date** (day/month/year) 22 December 1999 (22.12.99)

1. The following indications appeared on record concerning:

[X] the applicant [X] the inventor [ ] the agent [ ] the common representative

Name and Address	State of Nationality	State of Residence
KRAL, Vladimir	**	**
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [X] the nationality [X] the residence

Name and Address	State of Nationality	State of Residence
KRAL, Vladimir Na Kozacce 8/9275 120 00 Praha 1 Czech Republic	CZ	CZ
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[ ] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[ ] the elected Offices concerned
[ ] other:

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer S. De Michiel
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

WILSON, Mark, B.
Fulbright & Jaworski L.L.P.
600 Congress Avenue
Suite 2400
Austin, TX 78701
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year)
14 June 2000 (14.06.00)

Applicant's or agent's file reference
UTFK336P

IMPORTANT NOTIFICATION

International application No.
PCT/US99/30707

International filing date (day/month/year)
22 December 1999 (22.12.99)

1. The following indications appeared on record concerning:

[X] the applicant [X] the inventor [ ] the agent [ ] the common representative

Name and Address	State of Nationality	State of Residence
MARTASEK, Pavel	**	**
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [X] the nationality [X] the residence

Name and Address	State of Nationality	State of Residence
MARTASEK, Pavel 7531 Pipers Lane San Antonio, TX 78251 United States of America	CZ	US
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[ ] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[ ] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

S. De Michiel

Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY DOCKETING REQUIRED

From the INTERNATIONAL BUREAU

To:

WILSON, Mark, B.
Fulbright & Jaworski L.L.P.
600 Congress Avenue
Suite 2400
Austin, TX 78701
ETATS-UNIS D'AMERIQUE

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

Date of mailing (day/month/year) 31 January 2001 (31.01.01)	
Applicant's or agent's file reference UTFK336P	**IMPORTANT NOTIFICATION**
International application No. PCT/US99/30707	**International filing date** (day/month/year) 22 December 1999 (22.12.99)

1. The following indications appeared on record concerning:
[X] the applicant [X] the inventor [ ] the agent [ ] the common representative

Name and Address	
RECEIVED F & J AUSTIN INTL FEB 13 2001	State of Nationality: State of Residence: Telephone No.: Facsimile No.: Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:
[X] the person [ ] the name [ ] the address [ ] the nationality [ ] the residence

Name and Address	
LI, Huiying 384 Falling Star Irvine, CA 92614 United States of America	State of Nationality: CN State of Residence: US Telephone No.: Facsimile No.: Teleprinter No.:

3. Further observations, if necessary:
Additional applicant and inventor for US only.

4. A copy of this notification has been sent to:
[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland** Facsimile No.: (41-22) 740.14.35	Authorized officer C. Cupello Cupello Telephone No.: (41-22) 338.83.38

WO 00/37653
PCT/US99/30707

Pat. docketed

PATENT COOPERATION TREATY

P/R - WIM - NAK - STW

DOCKETED
DATE Pub. data entered
INITIALS C.L.

From the INTERNATIONAL BUREAU

PCT

To:
WILSON, Mark, B.
Fulbright & Jaworski L.L.P.
600 Congress Avenue
Suite 2400
Austin, TX 78701
ETATS-UNIS D'AMERIQUE

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

Date of mailing (day/month/year) 29 June 2000 (29.06.00)		
Applicant's or agent's file reference UTFK336P	**IMPORTANT NOTICE**	
International application No. PCT/US99/30707	**International filing date** (day/month/year) 22 December 1999 (22.12.99)	**Priority date** (day/month/year) 22 December 1998 (22.12.98)
Applicant BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM et al		

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AU,CN,JP,KP,KR,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   AE,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,CA,CH,CR,CU,CZ,DE,DK,DM,EA,EE,EP,ES,FI,GB,GD,GE,GH,GM,HR,HU,ID,IL,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MN,MW,MX,NO,NZ,OA,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,UZ,VN,YU,ZA,ZW
   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on 29 June 2000 (29.06.00) under No. WO 00/37653

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

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AUSTIN INTL
JUL 20 2000

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer J. Zahra
Facsimile No. (41-22) 740.14.35	Telephone No. (41-22) 338.83.38

PCT/US99/30707

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

INFORMATION CONCERNING ELECTED OFFICES NOTIFIED OF THEIR ELECTION

(PCT Rule 61.3)

To:

WILSON, Mark, B.
Fulbright & Jaworski L.L.P.
600 Congress Avenue
Suite 2400
Austin, TX 78701
ETATS-UNIS D'AMERIQUE

RECEIVED
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JAN 2 9 2001

Date of mailing (day/month/year) 15 January 2001 (15.01.01)		
Applicant's or agent's file reference UTFK336P	**IMPORTANT INFORMATION**	
International application No. PCT/US99/30707	**International filing date** (day/month/year) 22 December 1999 (22.12.99)	**Priority date** (day/month/year) 22 December 1998 (22.12.98)
Applicant BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM et al		

1. The applicant is hereby informed that the International Bureau has, according to Article 31(7), notified each of the following Offices of its election:

   AP :GH,GM,KE,LS,MW,SD,SL,SZ,TZ,UG,ZW
   EP :AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE
   National :AU,BG,CA,CN,CZ,DE,IL,JP,KP,KR,MN,NO,NZ,PL,RO,RU,SE,SK,US

2. The following Offices have waived the requirement for the notification of their election; the notification will be sent to them by the International Bureau only upon their request:

   EA :AM,AZ,BY,KG,KZ,MD,RU,TJ,TM
   OA :BF,BJ,CF,CG,CI,CM,GA,GN,GW,ML,MR,NE,SN,TD,TG
   National :AE,AL,AM,AT,AZ,BA,BB,BR,BY,CH,CR,CU,DK,DM,EE,ES,FI,GB,GD,GE,GH,GM,HR,HU,ID,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MW,MX,PT,SD,SG,SI,SL,TJ,TM,TR,TT,TZ,UA,UG,UZ,VN,YU,ZA,ZW

3. The applicant is reminded that he must enter the "national phase" **before the expiration of 30 months from the priority date** before each of the Offices listed above. This must be done by paying the national fee(s) and furnishing , if prescribed, a translation of the international application (Article 39(1)(a)), as well as, where applicable, by furnishing a translation of any annexes of the international preliminary examination report (Article 36(3)(b) and Rule 74.1).

   Some offices have fixed time limits expiring later than the above-mentioned time limit. For detailed information about the applicable time limits and the acts to be performed upon entry into the national phase before a particular Office, see Volume II of the PCT Applicant's Guide.

   The entry into the European regional phase is postponed **until 31 months from the priority date for all States** designated for the purposes of obtaining a European patent.

RECEIVED
Docketed:
NO DOCKETING REQUIRED

JAN 2 6 2001

Client: UTFK:336P
Attorney(s): WIM
Initials: Km

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland** Facsimile No. (41-22) 740.14.35	Authorized officer: R. E. Stoffel Telephone No. (41-22) 338.83.38